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Phenolic Compounds and Biological Activities of *Dichrostachys cinerea* L.

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ABSTRACT

Two new flavonoidal compounds in addition to eight known ones, as well as two phenolic acids (1→12) were isolated for the first time from the methanol successive extract of the aerial parts of *Dichrostachys cinerea* by fractionation on polyamide column chromatography and further purification on a Sephadex column. They were identified by studying their chromatographic, hydrolytic and spectral characters (UV, ^{1}H-NMR, ^{13}C-NMR, ESI). These compounds were identified as: (1) 4, 3', 4'- trihydroxyauron-6-*O*-galactopyranoside, (2) quercetin-3-*O*-apiosyl-3"*O*-gallate, (3) myricetin-3-*O*-rhamnopyranoside, (4) myricetin-3-*O*-glucopyranoside (5) quercetin-3-*O*-α-L-rhamnopyranosyl (1'"→6")-*O*-β-D-glucopyranoside, (6) quercetin-3-*O*-rhamnopyranoside, (7) quercetin-3-*O*-glucopyranoside, (8) quercetin-3-*O*-galactopyranoside, (9) apigenin, (10) kaempferol, (11) isovanillic acid, and (12) vanillic acid. The LD_{50} of the total ethanol extract was 6.9 g/kg body weight. The anti-ulcer and anti-inflammatory activities of the total ethanol extract and successive extracts (petroleum ether, chloroform, ethyl acetate, and methanol) of the aerial parts of *D. cinerea* were studied. The total ethanol extract (200 mg/kg body weight) and the chloroform successive extract (100 mg/kg body weight) were found to exhibit the highest anti-ulcer activity (71.5% protection), and anti-inflammatory activity (89.83% potency), respectively. The antioxidant activity measured by DPPH using electron spin resonance spectroscopy of some of the isolated compounds was evaluated. Compounds 3 and 4 having a myricetin nucleus were found to exhibit the highest scavenging activity (95.70 and 94.60%, respectively) at 1 mg/ml as compared with ascorbic acid.

Keywords: anti-inflammatory, antioxidant, antiulcer activities, column chromatography, flavonoids, phenolic acids, spectral characters

INTRODUCTION

The aim of the present study was to isolate and identify phenolic compounds from the methanol successive extract and evaluating the anti-ulcer and anti-inflammatory activities of the total ethanol and successive extracts of the aerial parts of *Dichrostachys cinerea*, as well as to evaluate the antioxident activity of some of the isolated compounds.

D. cinerea (Family Fabaceae) is a semi-deciduous to deciduous tree up to 7 m tall with very characteristic bi-coloured flowers (bottlebrush) and is native to South Africa (Kartesz). *D. cinerea* was reported to be a medicinal plant used in the traditional Indian system of medicine and the uses of the different organs of the plant were mentioned (Rulangaranga 1989). The bark of the plant is used to treat dysentery, tooth-aches and elephantiasis. The leaves are laxative and used to treat gonorrhoea, boils, stomache problems and can remove poison from snake-bites, as an aphrodisiac and as an astringent for scorpion bite. Root infusions are taken for leprosy, syphilis coughs, as an anthelmentic, purgative and strong diuretic. The plant is used as a veterinary medicine in India (Rulangaranga 1989).

A novel 3-*O*-acyl mesquitol and 2,3-*trans*-3',4',7,8-tetrahydroxyflavan-3-ol isolated from the aerial parts of *D. cinerea* were found to have free-radical scavenging and α-glucosidase inhibitory activities (Jagadeeshwar *et al.* 2003).

Joshi and Sharma (1974) isolated friedelin, friedlan-3β-ol, β-sitosterol and α-amyrin from the bark of *D. cinerea*, octacosanol and sitosterol from the heart wood, hentricontanol, β-amyrin and β-sitosterol from the leaves of the same plant. The coumarins imperatorin, marmesin and aesculetin were detected in the coumarin fraction of *D. cinerea* leaves while β-sitosterol and stigmasterol were detected in the unsaponifiable fraction of the leaves of the same plant (Khattab 1998).

MATERIALS

Plant material

The aerial parts of *D. cinerea* L. (leaves, stems and flowers) were obtained from the Orman Botanical Garden, Giza, Egypt. The plant was authenticated by Mrs. Terase Labib, Taxonomist of Orman Garden and confirmed by the Taxonomist, Dr. M. El-Gebaly, National Research Centre (NRC). A voucher specimen (No. 135-2003) was kept in the Herbarium of Pharmacognosy Department, NRC.

Experimental animals

Adult Sprague Dawley albino rats weighing 130-150 g and albino mice weighing 25-30 g were obtained from the Animal House Colony of the National Research Centre, Egypt. They were kept under the same hygienic conditions and well balanced diet and water.

All animal procedures were performed after approval from the ethics committee of the National Research Centre and in accordance with the recommendations for the proper care and use of Laboratory animals (NIH Publication No. 85-23, revised, 1985).

Drugs

Indomethacin (Indocid) (Kahira Pharm. Ind. Co. A.R.E., Cairo, Egypt) was used as a standard anti-inflammatory agent; carrageenan (Sigma-Aldrich Co., Cairo, Egypt), was used for the induction of acute inflammation in rats; ascorbic acid (El Nasr Pharmaceutical Co., Cairo, Egypt) as a standard antioxidant agent; 1,1-Diphenyl-2-picrylhydrazyl (DPPH) (Sigma-Aldrich Co.).

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Authentic samples

Authentic sugars, flavonoid aglycones and gallic acid were kindly obtained from the Phytochemistry and Plant Systematic Department, National Research Center.

Apparatus

1. Finnigan LCQ Advantage Max 2005 (LC/MS/MS) Mass Spectrometers, for determination of the molecular weight of flavonoids.
2. Ultra Violet (UV) -Visible Spectrophotometer, Spectro UV–VIS double beam UVD–3500 Spectrophotometer, Labomed, Inc., for measuring UV spectral data of the isolated compounds, in the range of (200-500 nm) in methanol and with different diagnostic shift reagents.
3. Nuclear Magnetic Resonance (NMR) Spectrophotometer JEOL EX-270 MHz NMR and 500 MHz (for determination of ^{1}H-NMR) and 75 and 125 MHz (for determination of ^{13}C-NMR).
4. Ultraviolet Lamp for localization of spots on paper.
5. Elexsys Bruker 500, operated at X-band frequency (Germany) for evaluation of the antioxidant activity (ESR).

Solvent systems

S1: Acetic acid: water (15: 85) (Stahl 1969).
S2: *n*-Butanol: acetic acid: water (4: 1: 5 upper layer) (Stahl 1969).
S3: Chloroform: methanol (9: 1) (Ahmad *et al*. 1993) (Stahl 1969).
S4: Phenol: water 80% (Nawwar *et al.* 1994).

METHODS

A. Isolation and identification of phenolic compounds

1. Paper chromatographic (PC) investigation of the methanol extract

The methanol extract was subjected to two dimensional paper chromatography using S1 and S2. The result revealed the presence of eight flavonoidal compounds (positive response towards $AlCl_3$, spray reagent; Stahl 1969).

2. Column chromatoghraphic isolation and purification of flavonoids

Fifty g of the dried methanol extract were dissolved in aqueous methanol (3: 1), then applied on the top of a column (150 × 10 cm) containing 250 g polyamide powder and eluted by step-wise gradient solvent systems of decreasing polarity starting from 100% water to 100% methanol. Fractions of 500 ml were collected and monitored by PC using two solvent systems, S1 and S2. Chromatograms were visualized under UV light before and after exposure to ammonia vapours and spraying with $AlCl_3$; similar fractions were combined and concentrated to dryness under reduced pressure to obtain five sub fractions. The major compounds were isolated and purified by chromatographing over a Sephadex LH-20 column, to afford compounds **1** to **12**.

3. Acid hydrolysis of the isolated compounds

Acid hydrolysis of the isolated compounds as well as identification of the aglycones and sugar moieties were carried out according to Mabry *et al*. (1970). The aglycones were identified by comparison with authentic samples on paper chromatograms using solvent systems S1 and S2, while the sugar moieties were identified by spotting on paper chromatograms alongside authentic sugars using solvent systems S2 and S4.

4. Identification of the isolated compounds

The isolated compounds were subjected to UV, ^{1}H-NMR and ^{13}C-NMR spectral analysis for elucidation of their structures (**Fig. 1**).

B. Investigation of biological activities

1. Acute toxicity test (LD_{50})

The total ethanol extract of aerial parts of *D. cinerea* was subjected to LD_{50} determination according to the method described by Miller and Tainter (1944).

Male albino mice (25-30 g) were divided into groups each of six animals. Preliminary experiments were done to determine the minimum dose that kills all animals (LD_{100}) and the maximum dose that fails to kill any animal. Several doses at equal logarithmmic intervals were chosen in between these two doses, each dose was injected into a group of six animals by subcutaneous injection. The mice were then observed for 24 hours and symptoms of toxicity and mortality rates in each group were recorded and LD_{50} was calculated.

2. Anti-ulcer activity test

The anti-ulcer activity was performed according to Corell *et al.* (1979) method. In this method, stomach ulcers in rats were induced by oral administration of indomethacin. The tested extracts were assessed for their anti-ulcer activity by examining their ability to reduce or prevent the number of stomach ulcers.

Sixty six adult male albino rats were divided into eleven groups, each of six animals. They were starved for 18 h but given water. One group was orally treated with 20 mg/kilogram body weight (kg bwt) indomethacin (positive control). The remaining groups were orally treated with 20 mg/kg bwt indomethacin in addition to two oral doses (100 and 200 mg/kg bwt) of the total ethanol and successive extracts of the plant. Four hours later, rats were sacrificed, their stomaches were removed, cut, along the greater curvature, mounted on a flat surface, and microscopically examined for the presence of any ulcerated areas. The number of ulcers was counted for each group. Results are illustrated in **Table 1** and **Fig. 2**.

3. Anti-inflammatory activity test

Paw swelling, or footpad oedema, is a convenient method for assessing inflammatory responses to antigenic challenges and irritants (Winter *et al*. 1962).

This model uses carrageenan as an irritant to induce paw oedema. Typically, tested materials are assessed for acute anti-inflammatory activity by examining their ability to reduce or prevent the development of carrageenan-induced paw swelling. Non-steroidal anti-inflammatory drugs (NSAIDs), such as indomethacin, reduce paw swelling in a dose-dependent manner to a maximum of 60%.

Seventy-two adult male albino rats, divided into twelve groups, each of six animals, were orally treated with 50 and 100 mg/kg bwt of each extract, indomethacin (positive control), and saline (negative control). One hour after oral administration, all animals were given a sub-plantar injection of 0.1 ml of 1% carrageenan solution in saline in the right hind paw and 0.1 ml saline in the left hind paw. Four hours after oral administration, the rats were sacrificed. Both hind paws were, separately, excised and weighed. Oedema % and oedema inhibition % were calculated according to the following equations, respectively.

$$\% \text{ Oedema} = \frac{(\text{wt. of right paw - wt. of left paw})}{\text{wt. of left paw}} \times 100$$

$$\% \text{ Oedema inhibition} = \frac{(\text{Mc - Mt})}{\text{Mc}} \times 100$$

where Mc = the mean oedema in control group, and Mt = the mean oedema in the drug-treated group.

Data were statistically analyzed using the student's *t*-test (Snedecor and Cochran 1971).

Results with $p < 0.01$ were considered statistically significant. Results are illustrated in **Table 2** and **Fig. 3**.

4. Antioxidant activity test

The antioxidant activity of some of the isolated compounds (**1**, **3**, **4**, **5**, **6**) were assessed by measuring the ability of each compound to scavenge the free radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH) using electron spin resonance (ESR) spectroscopy (Calliste *et al.* 2001).

10^{-3} M solution of DPPH in methanol was prepared. Five mg of each compound, as well as, ascorbic acid were separately dissolved in 5 ml methanol.

1 ml DPPH solution + 1 ml MeOH were mixed and measured, considered as the reference solution.

1 ml DPPH solution + 1 ml of the MeOH solution of each compound or ascorbic acid were mixed and measured immediately. Results are illustrated in **Table 3** and **Fig. 4**.

ESR signal of DPPH in MeOH appears at g = 2.006 characterizing the free radical. The decrease of this signal after mixing with the solution of each compound is taken as indication of the antioxidant activity, measured as a double integration area (DIA):

$$\% \text{ Activity} = \frac{[\text{DIA (DPPH)} - \text{DIA (DPPH + compound)}]}{\text{DIA (DPPH)}} \times 100$$

RESULTS AND DISCUSSION

A. The isolated phenolic compounds

4,3',4'-Triihydroxy-aurone-6-*O*-galactoside (aureusidin-6-*O*-galactoside) (1)

Yellow amorphous powder (10 mg), soluble in MeOH with R_f values of 0.31 (S2), 0.11 (S1). It appeared as an orange spot on PC under UV light, turning fluorescent orange red when fumed with NH_3 vapour or spraying with $AlCl_3$. Compound 1 yielded on complete acid hydrolysis aurone nucleus as an aglycone and galactose as the sugar moiety.

UVλmax, nm (MeOH): 255, 336sh, 402; (+NaOMe): 260, 331, 365, 447; (+$AlCl_3$): 257, 275, 322sh, 437; (+$AlCl_3$/HCl): 257, 275, 331sh, 400; (+NaOAc): 259, 335sh, 410; (+NaOAc/H_3BO_3): 262, 335, 435.

^{1}H-NMR (500 MHz, $DMSOd_6$): δ 7.37 (1H, d, *J* = 1.65 Hz, H2'), 7.15 (1H, dd, *J* = 8.4 Hz, 1.65, H-6'), 6.80 (1H, d, *J* = 8.4 Hz, H-5'), 6.38 (1H, s, benzylic), 6.13 (1H, s, H-7), 6.11 (1H, s, H-4), 5.05 (1H, d, *J* = 7.55 Hz, H-1").

^{13}C-NMR (125 MHz, DMSO-d_6): δ 178 (C-3), 168 (C-6), 163.9 (C-8), 147.7 (C-4'), 145.6 (C-2), 145 (C-3'), 125.3 (C-4), 124.2 (C-6'), 123.3 (C-1'), 118 (C-5'), 115.9 (C-5), 113.7 (C-9), 111.5 (C-2'), 112.6 (C-10 benzylic), 102.55 (C-1"), 94 (C-7), 75.9 (C-5"), 73.5 (C-3"), 71.4 (C-2"), 69 (C-4"), 61 (C-6").

UV spectral data of compound **1** showed that the absorbance of band I in methanol exceeds 400 nm indicating that compound **1** may be of aurone class. Aurone compounds are considered as minor flavonoids as reported by Harborne (1994).

A bathochromic shift (45 nm) in band I with increase in intensity relative to that of MeOH upon addition of NaOMe was indicative for free 4'-OH, as well as, a bathochromic shift in band II <5 nm) on addition of NaOAc referred to the absence of a free 6-OH group. Hypsochromic shift of 37 nm in band I with $AlCl_3$/HCl relative to $AlCl_3$ spectrum, and bathochromic shift of 33 nm in band I with NaOAc/H_3BO_3 relative to MeOH, confirmed the presence of ortho-dihydroxy group in B-ring (Mabry *et al*. 1970).

^{1}H-NMR spectrum showed the presence of aurone structure as indicated by the presence of a singlet at 6.38 ppm (Milovanovic *et al.* 2002). The ^{1}H-NMR data of the aglycone nucleus is identical with those previously reported for aureusidin (Harborne 1994).

From the above data and the product of hydrolysis it could be concluded that compound 1 is identified as 4,3',4'-triihydroxy-aurone-6-*O*-galactoside (aureusidin-6-*O*-galactoside).

Aureusidin (4, 6, 3', 4'-tetrahydroxy-aurone) was reported to occur in *Melanorrhoea aptera* (Anacardiaceae) (Harborne and Mabry 1982), aureusidin-6-*O*-glucoside was found in *Antirrhinum orontium* (Scrophulariaceae). Aureusidin 6-*O*-rhamnoside was reportedly found in *Pterocarpus marsupium* (Fabaceae) (Mohan and Joshi 1989).

Since compound **1** was not isolated before it could be considered as a new compound isolated for the first time from *D. cinerea* in the current study.

Quercetin-3-*O*-(3''-*O*-galloyl)-α-D-apiofuranoside (2)

Yellow amorphous powder (20 mg) soluble in MeOH with R_f values of 0.659 (S2) and 0.292 (S1). It appeared as a deep purple spot on PC under UV light, turning fluorescent yellow when fumed with NH_3 vapour or spraying with $AlCl_3$. It gave a dark blue colour with alcoholic $FeCl_3$. Compound **2** yielded on complete acid hydrolysis quercetin and gallic acid in the organic layer and the sugar apiose in aqueous phase.

UVλmax, nm (MeOH): 261, 295sh, 356; (+NaOMe): 271, 325, 410; (+$AlCl_3$): 275, 303, 425; (+$AlCl_3$/HCl): 272, 354, 400; (+NaOAc): 271, 318, 398; (+NaOAc/H_3BO_3): 271, 355, 398.

^{1}H-NMR (270 MHz, $DMSOd_6$): δ 7.50 (2H, m, H-2'/6'), 6.85 (2H, s, H-2'"/6'"), 6.82 (1H, dd, *J* = 9 Hz, H-5'), 6.40 (1H, d, *J* = 2.5 Hz, H-8), 6.19 (1H, d, *J* = 2.5 Hz, H-6), 5.55 (1H, s, H-1") 4.17 (2H, br. s, H-5"), 4.03 (1H, d, H-4"b), 3.73 (1H, d, H-4"a), 3.75 (1H, s, H-2").

^{13}C-NMR (67.5 MHz, $DMSOd_6$): δ 177.67 (C-4), 165.67 (C=O gallic), 164.31 (C-7), 161.33 (C-5), 157.56 (C-2), 156.52 (C-9), 148.53 (C-4'), 145.57 (C-3'", C-5'" gallic), 145.16 (C-3'), 138.66 (C-4'"gallic), 133.29 (C-3), 121.58 (C-1'), 121.03 (C-6'),119.14 (C-1'" gallic), 115.80 (C-2'), 115.66 (C-5'), 108.75 (C-2'",C-6'"gallic), 107.86 (C-1"), 104.11 (C-10), 98.84 (C-6), 93.80 (C-8), 80.00 (C-3"), 77.18 (C-2"), 73.68 (C-4"), 63.4 (C-5").

ESI-MS and ESI-MS fragmentation pattern, showed m/z 585, 432.96 and 301.

UV spectrum of compound **2** showed the behaviour of quercetin nucleus. It gave quercetin, gallic acid and apiose on complete acid hydrolysis. So it was assumed to be quercetin-3-*O*-galloyl apiose.

^{1}H-NMR spectrum of compound **2** afforded the characteristic signals of quercetin nucleus. Two doublets at δ 6.40 and 6.19 ppm assigned to H-6 and H-8 protons respectively and a multiplate at δ 7.50 ppm assigned to H-2' and H-6'. Besides these signals, another singlet at δ 6.85 ppm assigned to H-2'", 6'", of gallic acid moiety another doublet of doublet at 6.82 ppm for H-5' but overlapped by the singlet of H-2'" and H-6'" of gallic moiety. The down field shift of C-3" ((Δ ≅2 ppm) indicates that gallic acid attached to it.

The ^{13}C-NMR presented 25 signals of which 15 were attributed to the aglycone, 4 to the gallic acid moiety and 5 signals to the possible presence of apiofuranosyl unit (Agrawal 1989).

Also the ^{13}C-resonance of galloyl moiety at δ 108.75 for (C-2'", C-6'") and δ 145.57 for (C-3'", C-5'") was confirmative evidence for the presence of galloyl group. The attachment of the galloyl moiety to OH at C-3" of the sugar was deduced from the unchanged location of H-2" at δ (3.75) and H-4 " at δ 4.03 (d, H-4"b), δ3.79 (d, H-4"a) and H-5" at δ (4.17), as well as this evidence was confirmed from the downfield shift of C-3" at 80.00 ppm (Δ ≅ 2 ppm), besides the presence of a characteristic signal at 107.86 for (C-1")which is indicative for the presence of α-D apiose sugar. Also the structure was confirmed by determination of negative ESI-MS: -m/z 585 [M^+–H], 432.96 [M^+–H–galloyl] and 301[M^+–H–galloyl-apiose]. The ESI-MS fragmentation pattern shows the following fragments m/z 585 [M^+–H], 432 .96 [M^+–H–galloyl], 301[M^+–H–galloyl-apiose]. So, compound 2 was identified as quercetin-3-*O*-(3"-*O*-galloyl)-α-D-apiofuranoside, having the molecular formula ($C_{27}H_{22}O_{15}$). It is worth to mention that flavonoid apiofuranosides are rare in nature and compound **2** was not isolated before. So it could be concluded that compound **2** is a

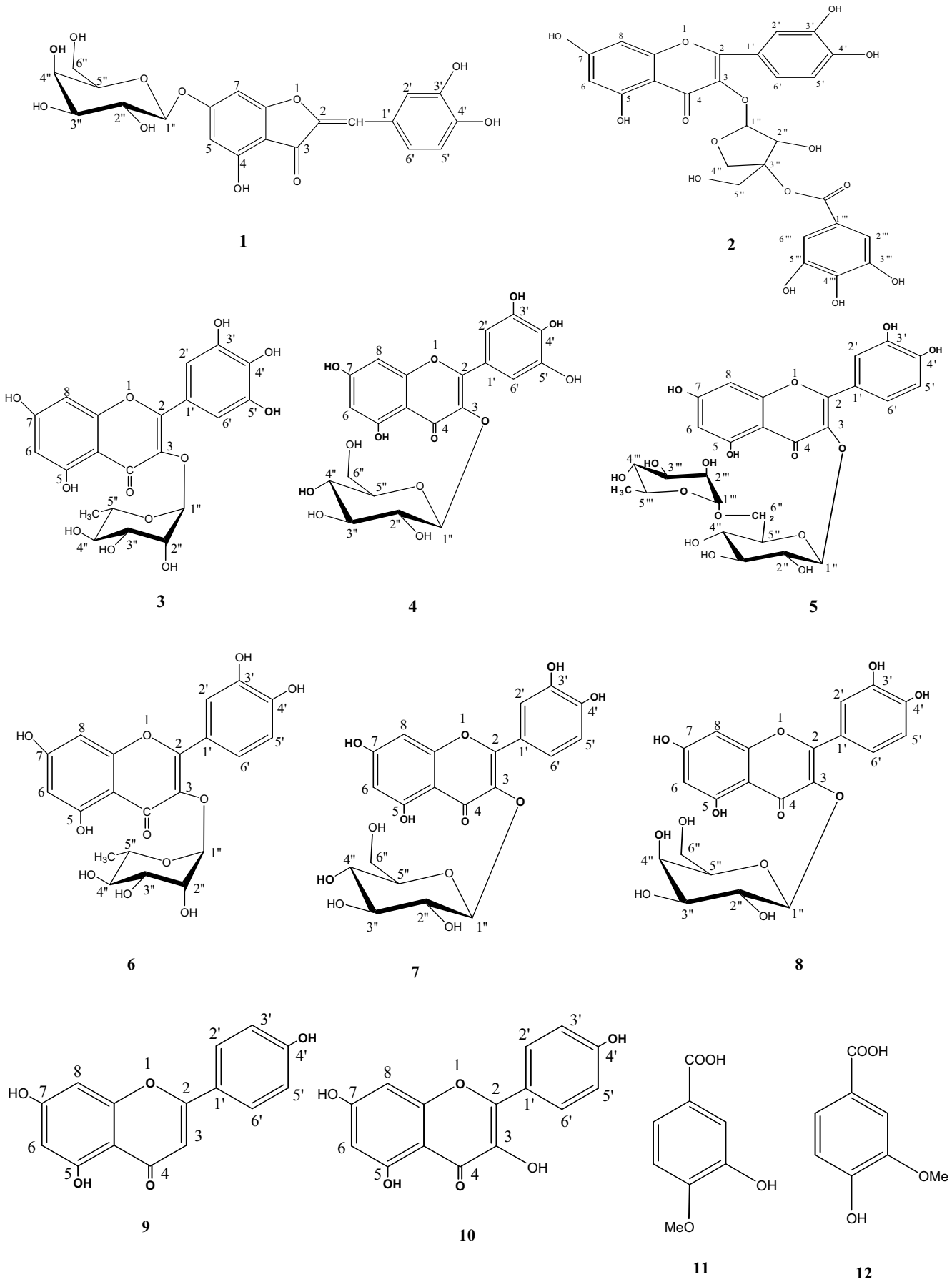

Fig. 1 Chemical structures of the compounds isolated from the aerial parts of *Dichrostachys cinerea* L. (compounds 1→12).

new compound, isolated for the first time from the aerial parts of *D. cinerea*.

Myricetin-3-*O*-α-L-rhamnopyranoside (3)

A yellow amorphous powder (18 mg) with R_f values of 0.60 (S2) and 0.42 (S1) soluble in MeOH and H_2O. It appeared

as a deep purple spot on PC under UV light, turning fluorescent yellow when fumed with NH_3 vapour or spraying with $AlCl_3$. It yielded myricetin as an aglycone and rhamnose as the sugar moiety on complete acid hydrolysis.

UVλmax, nm (MeOH): 256, 301, 353; (+NaOMe): 265, 398; (+$AlCl_3$), 272, 315, 433; (+$AlCl_3$/HCl): 272, 308sh, 354, 399; (+NaOAc): 270, 315, 371; (+NaOAc/H_3BO_3): 258, 298, 375.

^{1}H-NMR (500 MHz, $DMSOd_6$): δ 6.89 (2H, s, H-2'/6'), 6.2 (1H, d, *J* = 2.5 Hz, H-6), 6.37 (1H, d, *J* = 2.5 Hz, H-8), 5.2 (1H, br. s, H-1"), 3.90-3.20 (m, remaining sugar protons), 0.85 (3H, d, *J* = 6 Hz, H-6"). From R_f values and colours under UV of compound **3**, it could be concluded that it is a 3-substituted glycoside. From its UV spectral data, it could be concluded that it have myricetin nucleus (Mabry *et al*. 1970) and from its ^{1}H-NMR spectral signals, compound **3** could be identified as myricetin-3-*O*-α-L-rhamnopyranoside.

Myricetin-3-*O*-β-D-glucopyranoside (4)

Compound **4** (30 mg) showed R_f values of 0.42 (S2) and 0.25 (S1). It appeared as a deep purple spot on PC under UV light, turning fluorescent yellow when fumed with NH_3 vapour or spraying with $AlCl_3$. It yielded on complete acid hydrolysis myricetin as an aglycone and glucose as the sugar moiety.

UVλmax, nm (MeOH): 258, 303,350; (+NaOMe): 267, 395; (+$AlCl_3$): 272, 313, 433; (+$AlCl_3$/HCl): 272, 308sh, 353, 401; (+NaOAc): 275, 315, 375; (+NaOAc/H_3BO_3): 262, 310, 376.

^{1}H-NMR (500 MHz, $DMSOd_6$): δ 7.16 (2H, s, H-2'/6'), 6.13 (1H, d, *J* = 2.5 Hz, H-6), 6.35 (1H, d, *J* = 2.5 Hz, H-8), 5.45 (1H, d, *J* = 7.55 Hz, H-1"), 3.90-3.20 (m, remaining sugar protons).

^{13}C-NMR (125 MHz, $DMSOd_6$): δ 177.85 (C-4), 164.83 (C-7), 161.71 (C-5), 156.81 (C-2), 156.71 (C-9), 146.49 (C-3'), 145.87 (C-5'), 137.97 (C-4'), 133.95 (C-3), 120.49 (C-1'), 109 (C-2',6'), 104.37 (C-10), 101.4 (C-1"), 99.22 (C-6), 93.00 (C-8), 78.04 (C-5"), 77.04 (C-3"), 74.44 (C-2"), 70.36 (C-4") 61.52 (C-6").

From R_f values and colours under UV of compound **4**, it could be concluded that it is a 3-substituted glycoside. From its UV spectral data, it could be concluded that it had a myricetin nucleus (Mabry *et al*. 1970) and from its ^{1}H NMR and ^{13}C NMR spectral signals, compound **4** was identified as myricetin-3-*O*-β-D-glucopyranoside.

Quercetin-3-*O*-α-L–rhamnopyranosyl (1'''→6'')-*O*-β-D-glucopyranoside (rutin)(5)

Compound **5** obtained as yellow amorphous powder (100 mg) appeared as a deep purple spot on PC under UV light, turning fluorescent yellow when fumed with NH_3 vapour or spraying with $AlCl_3$ with R_f values of 0.38 (S2) and 0.66 (S1). It yielded quercetin as an aglycone, rhamnose and glucose as the sugar moieties on complete acid hydrolysis.

UVλmax, nm (MeOH: 258, 303, 350; (+NaOMe): 267, 395; (+$AlCl_3$): 272, 313, 433; (+$AlCl_3$/HCl): 272, 308sh, 353, 401; (+NaOAc): 275, 315, 375; (+NaOAc/H_3BO_3): 262, 310, 376.

^{1}H-NMR (500 MHz, $DMSOd_6$): δ 7.54 (2H, m, H-2'/6'), 6.85 (1H, d, *J* = 9 Hz, H-5'), 6.38 (1H, d, *J* = 2.5 Hz, H-8), 6.19 (1H, d , *J* = 2.5 Hz, H-6), 5.35 (1H, d, *J* = 7.5 Hz, H-1"), 5.33 (1H, br. s, aliphatic OH), 5.02, (2H each, br. s, aliphatic OH groups), 4.39 (1H, s, H-1'"), 3.90-3.20 (m, remaining sugar protons), 0.99 (3H, d, J = 6 Hz, H-6'").

^{13}C-NMR (125 MHz, $DMSOd_6$): δ 177.85 (C-4), 164.70 (C-7), 161.68 (C-5), 157.14 (C-2), 156.95 (C-9), 148.92 (C-4'), 145.25 (C-3'), 133.76 (C-3), 122.12 (C-6'), 121.66 (C-1'), 116.73 (C-2'), 115.72 (C-5'), 104.41 (C-10), 101.66 (C-1'"), 101.23 (C-1"), 99.24 (C-6), 94.16 (C-8), 74.58 (C-3"), 72.33 (C-5"),72.2 (C-4'"), 71.05 (C-2"), 70.8 (C-2'"), 70.87 (C-3'"), 70.49 (C-4"), 68.74 (C-6"), 18.19 (C-6'").

From UV spectral data, compound **5** was suggested to have a quercetin nucleus (Mabry *et al.* 1970). In the aliphatic region of ^{13}C-NMR 12 carbon resonances assigned for a rutinoside moiety among which the most downfield signals at δ 101.23 and 101.66 assigned to the two anomeric carbons C-1" and C-1'", respectively. Furthermore, the attachment of the α-rhamnosyl moiety was confirmed on C-6" depending on the characteristic α-downfield shift of its ^{13}C-resonance to 63.00 (Δ ≈ +3 ppm) and β-upfield of C-5"at 72.33 (Δ ≈ -1.5 ppm) confirm the 1'"→6" interglycosidic linkage. From all the above mentioned data compound **5** was identified as quercetin-3-*O*-α-L-rhamnopyranosyl (1'"→6")-*O*-β-D-glucopyranoside (Rutin).

Quercetin-3-*O*-α-L-rhamnopyranoside (6)

Compound **6** exhibited R_f values of 0.69 (S2) and 0.62 (S1). It yielded on complete acid hydrolysis quercetin as an aglycone and rhamnose as the sugar moiety.

UVλmax, nm (MeOH): 256, 298sh, 351; (+NaOMe): 271, 326sh, 398; (+$AlCl_3$): 275, 304, 330, 422; (+$AlCl_3$/HCl): 269, 300sh, 353, 398; (+NaOAc): 269, 317, 362; (+NaOAc/H_3BO_3): 259, 299, 378.

^{1}H-NMR (500 MHz, $DMSOd_6$): δ 7.26 (2H, m, H2'/6'), 6.83 (1H, d, *J* = 9 Hz, H-6/5'), 6.49 (1H, d, *J* = 2.5 Hz, H-8), 6.14 (1H, d, *J* = 2.5 Hz, H-6), 5.25 (1H, br. s, H-1"), 0.78 (3H, d, *J* = 6 Hz, CH_3).

^{13}C-NMR (67.5 MHz, $DMSOd_6$): δ 177.42 (C-4), 167.45 (C-7), 161.40 (C-5), 157.01 (C-2), 157(C-9), 149.19 (C-4'), 145.57 (C-3'), 134.12 (C-3), 131.97 (C-6'), 121.40 (C-1'), 115.71 (C-2'), 115.40 (C-5'), 103.10 (C-10), 101.97 (C-1"), 99.98 (C-6), 94.47 (C-8) ,71.47 (C-4"), 70.94, 70.85, 70.62 (C-2",C-5",C-3"), 17.78 (C-6").

From ^{1}H-NMR and ^{13}C-NMR compound **6** could be identified as quercetin-3-*O*-α-L-rhamnopyranoside.

Quercetin-3-*O*-β-D-glucopyranoside (7)

Compound **7** (20 mg) showed R_f values of 0.58 (S2) and 0.44 (S1). It yielded on complete acid hydrolysis quercetin as an aglycone and glucose as the sugar moiety.

UVλmax, nm (MeOH): 257, 295sh, 357; (+NaOMe): 272, 328sh, 410; (+$AlCl_3$): 274, 303, 421; (+$AlCl_3$/HCl): 269, 359, 402; (+NaOAc): 270, 321sh, 369; (+NaOAc/H_3BO_3): 261, 298, 378.

^{1}H-NMR (500 MHz, $DMSOd_6$): δ 7.58 (2H, m, H-2'/6'), 6.83 (1H, d, *J* = 9 Hz H-5'), 6.35 (1H, d, *J* = 2.5 Hz, H-8), 6.15 (1H, d, *J* =2.5 Hz, H-6), 5.46 (1H, d, *J* = 7.55 Hz, H-1"), 3.2-3.8 (m, sugar protons).

^{13}C-NMR (125 MHz, $DMSOd_6$): δ 177.83 (C-4), 162.14 (C-7), 161.73 (C-5), 157.38 (C-2), 156.89 (C-9), 149.11 (C-3`), 148.47 (C-4'), 145.38 (C-5'), 133.77 (C-3), 121.61 (C-1'), 116.65 (C-2') 115.72 (C-6'), 104.21 (C-10), 101.44 (C-1"), 99.37 (C-6), 94.12 (C-8), 78.08 (C-5"), 77.03 (C-3"), 74.62 (C-2"), 70.45 (C-4"), 61.48 (C-6"). So compound **7** was identified as quercetin-3-*O*-β-D-glucopyranoside.

Quercetin-3-*O*-galactopyranoside (8)

Compound **8** (20mg) showed R_f values of 0.61 (S2) and 0.36 (S1). It yielded on complete acid hydrolysis quercetin as an aglycone and glactose as the sugar moiety.

UVλmax, nm (MeOH): 256, 294, 357; (+NaOMe): 271, 327, 408; (+$AlCl_3$): 273, 304, 423; (+$AlCl_3$/HCl): 268, 360sh, 402; (+NaOAc): 269, 326, 370; (+NaOAc/H_3BO_3): 261, 297sh, 378.

^{1}H-NMR (500 MHz, $DMSOd_6$):δ 7.58 (2H, m, H-2'/6'), 6.83 (1H, d, *J* = 9 Hz, H-5'), 6.41 (1H, d, *J* = 2.5 Hz, H-8), 6.20 (1H, d, *J* = 2.5 Hz, H-6), 5.46 (1H, d, *J* = 7.5 Hz, H-1"), 3.2-4 (remaining sugar proton).

Compound **8** was identified as quercetin-3-*O*-galactopyranoside.

Apigenin (9)

Compound **9** was found to be an aglycone in nature, it was

obtained as a yellow powder (15 mg) soluble in MeOH with R_f values of 0.78 (S2) and 0.01 (S1). It appeared as a deep purple spot on PC under UV light, turning fluorescent yellow when fumed with NH_3 vapour or spraying with $AlCl_3$.

UVλmax, nm (MeOH): 269, 298, 334; (+NaOMe): 276, 322, 394; (+$AlCl_3$): 276, 302, 346, 382; (+$AlCl_3$/HCl): 276, 300, 340, 382; (+NaOAc): 280, 300, 368; (+NaOAc/H_3BO_3): 269, 297, 337. The UV spectral data showed two major UV absorption bands in MeOH, band I at 334 nm and band II at 269 nm, typical for a flavone nucleus. Compound **9** showed the behaviour of apigenin with shift reagents.

^{1}H-NMR (270 MHz, $DMSOd_6$): δ 7.94 (2H, d, *J* = 8.1, H-2′/6′), 6.93 (2H, d, *J* = 8.1 Hz, H-3′/5′), 6.79 (1H, s, H-3), 6.48 (1H, d, *J* = 2.5 Hz, H-8), 6.18 (1H, d, *J* = 2.5 Hz, H-6). ^{1}H-NMR data confirmed the presence of apigenin. It showed the four aromatic protons of ring B at δ 7.94 and 6.93, the two aromatic protons of ring A at δ 6.48 and 6.18. So, compound **9** was identified as apigenin.

Kaempferol (10)

Compound **10** was found to be an aglycone in nature it was obtained as a yellow powder (18 mg) soluble in MeOH with R_f values of and 0.74 (S2) and 0.11 (S1). It appeared as a yellow spot on PC under UV light, turning fluorescent yellow when fumed with NH_3 vapour or sprayed with $AlCl_3$.

UVλmax, nm (MeOH): 266, 297sh, 363; (+NaOMe): 284, 330, 421; (+$AlCl_3$): 265, 275, 308, 322, 350, 419; (+$AlCl_3$/HCl): 265, 275, 304, 350, 419; (+NaOAc): 273, 283, 336; (+NaOAc/H_3BO_3): 266, 298, 347, 373.

^{1}H-NMR (270 MHz, $DMSOd_6$): δ 8.05 (2H, d, *J* = 8.1, H-2′/6′), 6.93 (2H, d, *J* = 8.1 Hz, H-3′/5′), 6.43 (1H, d, *J* = 2.5 Hz, H-8), 6.18 (1H, d, *J* = 2.5 Hz, H-6).

^{1}H-NMR of compound **10** confirmed the presence of kaempferol, as it showed two signals of the four aromatic protons of ring B at δ 8.05 and 6.93 and two signals of the two aromatic protons of ring A at δ 6.43 and 6.18. So, compound **10** was identified as kaempferol.

3-Hydroxy-4-methoxy-benzoic acid (isovanillic acid) (11)

Compound **11** was obtained as a white amorphous powder (10 mg) showing a violet spot under short UV light. It gave dark green colour with $FeCl_3$ spray reagent and R_f values of 0.68 (S2), 0.77 (S1) and 0.46 (S3).

UVλmax, nm (MeOH): 271; (+NaOMe): 271.

^{1}H-NMR (500 MHz, $DMSOd_6$): δ 8.81 (1H, br, s, OH carboxylic), 8.56 (1H, br. s, OH-3) 7.53 (1H, s, H-2), 7.12 (1H, dd, *J* = 2.5, *J* = 9.2 Hz, H-6), 7.06 (1H, d, *J* = 9.2 Hz, H-5), 3.64 (3H, s, OCH_3). UV spectral data of compound **11** did not give shift with NaOMe. So compound **11** was identified as 3-hydroxy-4-methoxy-benzoic acid (isovanillic acid).

3-Methoxy-4-hydroxy-benzoic acid (vanillic acid) (12)

Compound **12** (10 mg) showed the same characters of compound **11**.

UVλmax, nm (MeOH): 258, 290; (+NaOMe): 281, 296.

^{1}H-NMR (270 MHz, DMSO-d_6): δ 8.82 (1H, s, OH carboxylic), 8.54 (1H, s, OH-4) 7.50 (1H, s, H-2), 7.12 (1H, dd, *J* = 2.5, 9.2 Hz, H-6), 7.04 (1H, d, *J* = 9.2 Hz, H-5), 3.63 (3H, s, OCH_3). UV spectral data of compound **12** gave two absorption maxima, so it has a free OH group at position 4 (Harborne 1984). Compound **12** was identified as 3-methoxy-4-hydroxy-benzoic acid (vanillic acid).

B. Biological activities

1. Acute toxicity (LD_{50})

The LD_{50} of the total ethanol extract of the aerial parts of *D. cinerea* was found to be 6.9 g/kg bwt, so the plant is safe to be used by animals.

2. Anti-ulcer activity

Anti-ulcer activity of the different extracts of the aerial parts the plant (**Table 1, Fig. 2**) showed that the highest activity was exhibited by 200 mg of the total ethanol extract (71.5% protection) followed by 100 mg of total ethanol (70.7% protection) in comparison with indomethacin (0% protection).

3. Anti-inflammatory activity

A significant inhibition of the rat paw oedema weight induced by carrageenan was exhibited by some extracts. The highest activity was exhibited by 100 mg of chloroform extract (89.83% potency) followed by by 100 mg of total ethanol extract (88.84% potency) in comparison with indomethacin (100% potency) (**Table 2, Fig. 3**).

4. The antioxidant activity

Antioxidant activity of some of the isolated compounds from *D. cinerea* are presented in **Table 3** and **Fig. 4**, which revealed that compounds 3 and 4 having a myricetin nucleus exhibited the highest scavenging activity (95.70 and

Table 1 Anti-ulcer activity of total ethanol and successive extracts of the aerial parts of *Dichrostachys cinerea* on male albino rats (*n* = 6).

Group	Dose (mg/kg b.wt.)	Number of gastric ulcers (Mean ± S.E.)	% Protection
Indomethacin +	100	3.6 ± 0.2	70.7
ethanol	200	3.5* ± 0.1	71.5
Indomethacin +	100	13.2* ± 0.8	28.3
petroleum ether	200	8.4* ± 0.1	54.9
Indomethacin +	100	10.9* ± 0.2	40.8
chloroform	200	6.8* ± 0.1	63.0
Indomethacin +	100	10.2* ± 0.4	44.6
ethyl acetate	200	6.4* ± 0.3	65.2
Indomethacin +	100	9.1* ± 0.1	50.5
methanol	200	5.7* ± 0.3	54.3
Indomethacin	20	18.4 ± 0.7	--

*Significantly different from the control at p<0.01

Table 2 Anti-inflammatory activity of total ethanol and successive extracts of the aerial parts of *Dichrostachys cinerea* and indomethacin on male albino rats (n=6).

Group	Dose (mg/kg b.wt.)	% of oedema (Mean ± S.E.)	% of inhibition	% Potency
Control	1 ml saline	60.7 ± 2.6	00	---
Total ethanol	50	39.4 ± 1.9	35.09	54.06
	100	25.7* ± 1.3	57.66	88.84
Petroleum	50	48.8 ± 2.3	19.6	30.20
ether	100	29.5* ± 0.7	51.4	79.19
Chloroform	50	43.1 ± 2.5	29.0	44.68
	100	25.3* ± 0.6	58.3	89.83
Ethyl acetate	50	45.8 ± 2.4	24.6	37.90
	100	33.5* ± 1.5	44.8	69.02
Methanol	50	39.2 ± 1.4	35.4	54.54
	100	28.7.1* ± 1.1	52.7	81.20
Indomethacin	20	21.3 ± 0.7	64.90	100

*Significantly different from the control at p<0.01

Table 3 Antioxidant activity of some of the isolated compounds from the aerial parts of *Dichrostachys cinerea*.

Compound	Concentration (mg/ml MeOH)	Double integration area (DIA)	% Activity
DPPH	10^{-3} M	379.67	00
Ascorbic acid	1 mg/ml	0	100
1	1 mg/ml	318.04	16
3	1 mg/ml	10.0	95.70
4	1 mg/ml	20.54	94.60
5	1 mg/ml	150.93	60.25
6	1 mg/ml	217.10	42.80

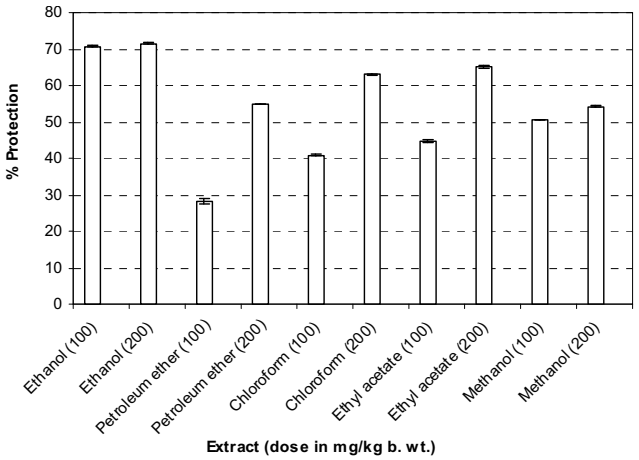

Fig. 2 Anti-ulcer activity of total ethanol extract and successive extracts (petroleum ether, chloroform, ethyl acetate and methanol) of the aerial parts of *Dichrostachys cinerea* using two dose levels (100 and 200 mg/kg b.wt.) (*n* = 6 rats), using indomethacine as ulcerogenic agent. Number of ulcers are counted after four hours. Values represent mean ± standard error.

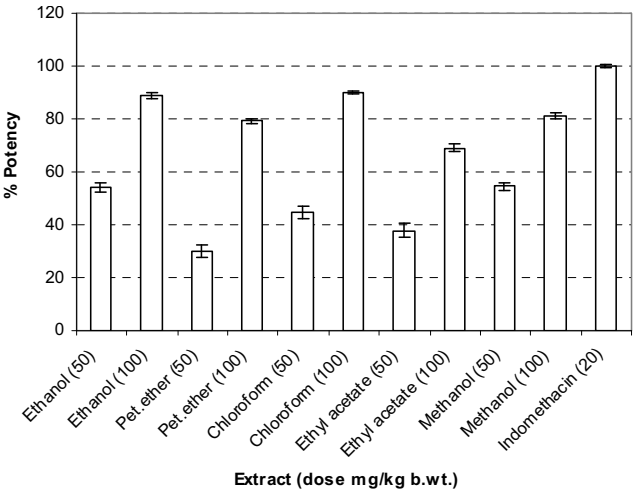

Fig. 3 Anti-inflammatory activity of total ethanol extract and successive extracts (petroleum ether, chloroform, ethyl acetate and methanol) of the aerial parts of *Dichrostachys cinerea* using two dose levels (50 and 100 mg/kg b.wt.) (*n* = 6 rats), using indomethacine as a standard anti-inflammatory agent. Oedema % was calculated after four hours of injection of 1% carrageenan solution. Values represent mean ± standard error.

94.60%, respectively) at the concentration of 1mg/ml as compared with ascorbic acid, followed by compound **5** (rutin) with 60.25% activity while compound **1** showed the lowest activity (16%). The antioxidant activity exhibited by the isolated compounds is attributed to their phenolic nature which was reported to have redox properties that allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers (Evans and Diplock 1995). Most interest has been devoted to the antioxidant activity of flavonoids, which is reported to be due to their ability to reduce free radical formation and to scavenge free radicals (Pietta 2000). With references to health beneficial principle of herbal products, poly phenols have been shown to represent effective antioxidant constituents (Rice-Evans 2001; Latte and Kolodziej 2004).

CONCLUSION

From a review of the literature of *D. cinerea*, it appeared that the plant was used in Indian folk medicine by patients for treatment of many diseases. This is in agreement with the high value of LD_{50} obtained by our study, so it is safe to be used. The anti-ulcer activity exhibited by the different extracts of the plant in the current study is in agreement with what was reported about the use of the leaves of the plant in treatment of stomach problems (Rulangaranga 1989).

It was reported that there is a linear relationship between the antioxidant capacities and the total phenolic content of medicinal herbs (Zheng and Wang 2001). The major antioxidant composition of phenolics were found to be flavonoid in nature (Siddhuraju and Becker 2003). This explains the good anti-oxidant activity exhibited by the tested

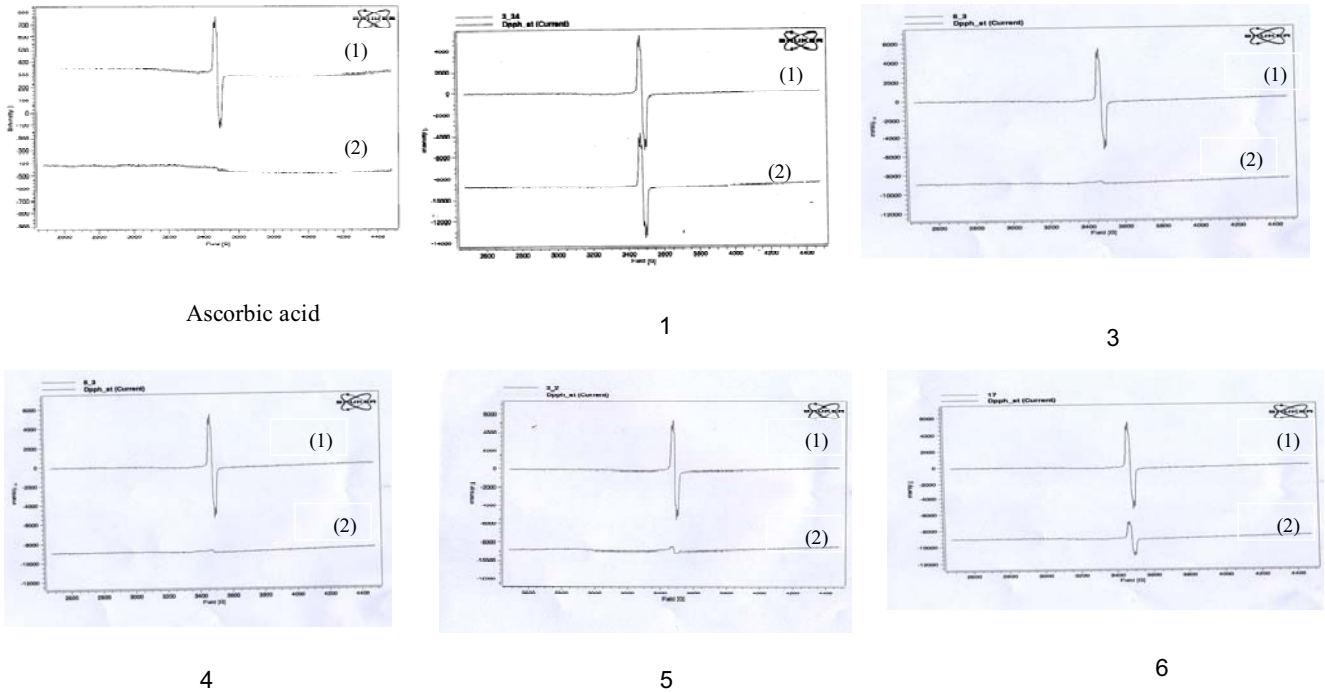

Fig. 4 Anti-oxidant activity of compounds 1, 3, 4, 5, 6 as compared with ascorbic acid (standard anti-oxidant agent) applying ESR method using DPPH as a source of free radical. (1) DPPH; (2) DPPH +1 mg/ml of compound. Results are measured as the decrease in the double integration area. X-axis = Field; Y-axis = Intensity.

compounds in the current study.

It has been reported that most flavonoids which exhibit a remarkable antioxidant activity possess also an anti-inflammatory activity (Cook and Sammans 1996). So the anti-inflammatory activity exhibited by the polar extracts may be attributed to the presence of many flavonoids in the polar extracts.

It may be concluded that the plant under investigation could be used as anti-ulcer, anti-inflmmatory and as antioxidant agent, so it is recommended to use the plant by patients after carrying out the clinical studies.

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