CHEMICAL CONSTITUENTS AND BIOLOGICAL ACTIVITY FROM CHLOROFORM EXTRACT OF ZILLA SPINOSA

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ABSTRACT

Six known compounds, bergaptene, psoralene, umbelliferone, 8-amyrin, friedelane and sterol-β-D-glucopyranoside in addition to Campesterol, Spinasterol, β-Sitosterol and Stigmasterol were isolated and identified from chloroform extract of the aperial parts of Zilla spinosa. Air-dried powder of the aerial parts of Zilla spinosa was extracted with petroleum ether (40-60°C) and the solvent was stripped off under reduced pressure. The above lipoidal matter was subjected to saponification for subsequent investigation. GLC analysis of the unsaponifiable matter from petroleum ether extract revealed series of hydrocarbons ranging from C16 to C30 in addition to Campesterol, Spinasterol, β-Sitosterol, α-Amyrine, β-Amyrine, Squalene, and Stigmasterol. Fatty acids analysis by using GLC technique revealed the presence of nine fatty acids. The anti-inflammatory activity of CHCl3 extract from the aerial parts of Zilla spinosa was assayed in male rate using carragenan-induced rat paw edema in comparison with Indomethacin. Analytical activity of CHCl3 extract of Zilla spinosa was assayed in mice using acetic acid. The crude drugs were maximally active at dose (400mg/kg b.wt). Biological screening of the chloroform extract showed that the plant is highly safe and has analgesic, anti-inflammatory, and anti-microbial effects. The chloroform extract was tested against two human tumor cell lines namely: colon (HCT116) and liver ( HepG2). The extract showed the more active against colon (HCT116) cell line IC50 14.5 ug/ml.

Keywords: Crucifereae, Zilla spinosa, Coumarins, Terpenes, Analgesic, Anti-inflammatory, cytotoxicity.

INTRODUCTION

Crucifereae is a large family of 3709 species in 375 genera [1], it is also known as the Brassicaceae. It was represented in Egypt by 53 genera and 105 species. It includes vegetable crops, some species were used as food and medicinal plants. They are used as antidiabetic, antibacterial [2], antifungal, antiancancer [3], antiinflammatory [4], and show a potent insecticidal effect [5]. Zilla spinosa is one of the most common plant species of family Crucifereae, due to its important uses in the folk medicinal; it's used as a drink against kidney stones or the gall bladder stones [6]. The genus Zilla was represented in Egypt by one species; it is annual, shrub, hispid with stiff hairs or spiny. Zspinosa was found to contain glucosinolates of free sinapine, progoitrin [7] and goitrin [8] which have antithyroid effect. Preliminary phytochemical study of Zspinosa growing in Egypt showed that it contains flavonoids, triterpenes and sterols [9]. Knowledge of the biological activities and or chemical constituents of plants are desirable, not only for the discovery of therapeutic agents, but also because such information may be of value in disclosing new sources of material.

MATERIAL AND METHODS

Plant Material

A fresh sample of Zilla spinosa was collected on Suez- Cairo desert road (Egypt) in April 2008. The sample was identified by Prof.Dr.Salwa A.Kawashy. The aerial parts of the plant were air dried and ground into fine powder.

Isolation of Lipids [10]

Preparation of Petroleum Ether Extract

Air-dried powder of the aerial parts of Zilla spinosa (50g) was extracted with petroleum ether (40-60°C) in continuous extraction apparatus. The solvent was stripped off under reduced pressure gave (8 g) residue. The above lipoidal matter was subjected to saponification for subsequent investigation of both unsaponifiable and saponifiable fractions.

Preparation of unsaponifiable matter and fatty acids

The above light petroleum extract was refluxed with alcoholic potassium hydroxide (10%) for 2 h, after stripping off ethanol and dilution with water, the unsaponifiable matter was extracted with chloroform. The residue left after evaporation of chloroform (U.S.M) was weighed (6.89 g) and kept for further investigation. Samples of the unsaponifiable fraction were subjected to GLC analysis (Agilent technology is 6890N Net work GS system USA, Centeral lab).

Preparation of fatty acids methyl ester

The soapy aqueous layer in each case, was acidified with 10% hydrochloric acid and the liberated fatty acids were extracted with ether. The residue left after evaporation of ether was weighed (0.64 g) and kept for further study (TFA). Total fatty acids were subjected to methylation [11].

Preparation of chloroform extract

The aerial parts of Z. spinosa (2kg) were defatted with petroleum ether (40-60°C) and extracted three times with 70% aqueous EtOH. Evaporation of the solvent under pressure from the combined extract afforded ethanolic extract (180 g). The extract was suspended in H2O and partitioned with CHCl3 and removal of the solvent afforded the CHCl3 extract (30 g). The CHCl3 extract was chromatographed on silica gel column chromatography with a gradient of n-hexane, n-hexane- chloroform (9:1 to 1:1) and chloroform–methanol (100:1 to 1:1) to yield fraction A (1.5 g) eluted with n-hexane, fr. B (1.3 g) 30% MeOH–hexane fr. C (0.8 g) 50% MeOH–hexane and fr. D (0.6 g) MeOH. The n-hexane fraction (1.5 g) were used for the separation of coumarins and sterols on silica gel column(G60,Merck) eluted with n-hexane and EtOAc in the proportion (9:1, 1:1 and 1:9), EtOAc and 0.5, 1, 2% methanol in EtOAc. similar fractions were combined together; chromatoplates were visualized under UV at 258 nm using solvent system, benzene: ethyl acetate(82) and 1:1KI spraying reagent for identification of coumarins and chlorosulphonic acid reagents for identification of sterols. Further purification of fr. B afforded 4 (2 mg), Fr. C afforded 5 (0.5mg), Fr. D yielded 6 (3 mg). Compound 6 was hydrolyzed and gave aglycone and sugar moiety. The resulting aglycon and sugar were characterized by standard procedure.[12].

The isolated compounds were identified by co-TLC with authentic samples and by comparison of their spectral data with those reported.

Biological Activity of the structures of the isolated: antimicrobial, analgesic, and anti-inflammatory activities tests were carried out in the biological section of Chemistry of Medicinal plants Department, NRC.

Anti-inflammatory activity

This method depends on induction of the left hind paw of rats by...
carrageenan [1% w/v][13]. Twenty four rats were divided into 4 groups. The 1st group was kept as a control. The 2nd and 3rd groups were orally administered (200 and 400 mg/kg b.wt) respectively, 1 hour before carrageenan injection. The last group was administered indomethacin (Indocid) in a dose of 10 mg/kg b.wt. orally as a standard reference.

The paw volume of each rat was measured using plethymometer; before carrageenan injection and then hourly for 4 hours post administration of the plant extract.

The edema rate and inhibition rate of each group were calculated as follows:

Edema rate (E) % = [Vt – Vo]/Vo
Inhibition rate (I) % = [Ec – Et] / Ec

Where:
Vo is the volume before carrageenan injection (ml).
Vt is the volume at t hour after carrageenan injection (ml).
Ec is the edema rate of the control group.
Et is the edema rate of the treated group.

**Analgesic effect:**

The analgesic effect of the tested extract was compared with that of the standard acetyl salicylic acid by using Writhing test. An acetic acid-induced abdominal constriction in mice (Writhing effect) was determined by the method described by [14]. Twenty four mice were divided into 4 equal groups and pre-treated as follows: group I which served as a control, was orally received distilled water in appropriate volumes. Groups II and III were received the aqueous solution of unknown at oral doses of 200 and 400 mg/kg b.wt. Respectively. Group IV was orally received acetyl salicylic acid in a dose of 100 mg/kg b.wt. After 30 minutes, each mouse was administered 0.7% of an aqueous solution of acetic acid (10 ml/kg b.wt) and the mice were then placed in transparent boxes for observation. The number of writhes was counted for 20 min after acetic acid injection. The number of writhes in each treated group was compared to that of a control un-treated group. The number of writhings and stretchings were recorded and the percentage protection was calculated using the following ratio:

Percentage of protection = [(Control mean-Treated mean) / Control mean] x 100

**Anti-microbial effect**

Antimicrobial activity of the tested samples was determined using a modified Kirby-Bauer disc diffusion method [15]. Briefly, 100 µl of the test bacteria / fungi were grown in 10 ml of fresh media until there reached a count of approximately 10⁸ cell/ml for bacteria or 10⁵ cell/ml for fungi [16]. About 100 µl of microbial suspension was spread onto agar plates corresponding to the broth in which they were maintained. Isolated colonies of each organism that might be playing a pathogenic role should be selected from the primary agar plates and tested for susceptibility by disc diffusion method [17]. Of the many media available, NCCLS recommends Mueller-Hinton agar because it results in good batch-to-batch reproducibility.

Disc diffusion method for filamentous fungi tested by using approved standard method (M38-A) developed by [18] for evaluating the susceptibilities of filamentous fungi to antifungal agents.

Disc diffusion method for yeasts developed by using approved standard method (M44-P) by the (NCCLS, 2003) [19].

Plates were inoculated with filamentous fungi as Aspergillus flavus at 25°C for 48 h; Gram (+) bacteria as Staphylococcus aureus and Bacillus subtilis and Gram (-) bacteria as Escherichia coli, Pseudomonas aeruginosa. They were incubated at 35-37°C for 24-48 h and then the diameters of the inhibition zones were measured in millimeters [15]. Standard discs of Tetracycline (antibacterial agent), Amphotericin B (Antifungal agent) served as positive controls for antimicrobial activity but filter discs impregnated with 10 µl of solvent (distilled water, chloroform, DMSO) were used as a negative control.

The agar used is Mueller-Hinton agar that is rigorously tested for composition and pH. Further the depth of the agar in the plate is a factor to be considered in the disc diffusion method. This method is well documented and standard zones of inhibition have been determined for susceptible and resistant values.

Blank paper disks (Schleicher & Schuell, 5 pain) with a diameter 8.0 mm were impregnated 10 µ of tested concentration of the stock solution.

When a filter paper disc impregnated with a tested chemical is placed on agar the chemical will diffuse from the disc into the agar. This diffusion will place the chemical in the agar only around the disc. The solubility of the chemical and its molecular size will determine the size of the area of chemical infiltration around the disc. If an organism is placed on the agar it will not grow in the area around the disc if it is susceptible to the chemical. This area of no growth around the disc is known as a Zone of inhibition (Clear zone).

For the disc diffusion, the zone diameters were measured with sliding calipers of the National Committee for Clinical Laboratory Standards[20]. Agar-based methods such as Eust and disc diffusion can be good alternatives because they are simpler and faster than broth-based methods [21].

**Cytotoxicity Activity**

**Cell lines**

Human tumor cell lines culture, HCT116 (colon carcinoma human cell line) and HepG2 (liver carcinoma human cell line). They were obtained frozen in liquid nitrogen from the (180 °C) American Type Culture Collection. The tumor cell lines were maintained in the National Cancer Institute, Cairo, Egypt, by serial sub-culturing.

**Culture Media**

HCT116 and HepG2 cells were suspended in RPMI 1640 medium (Sigma Chemical Co., St. Louis, Mo, and U.S.A) supplemented with 10 % fetal bovine serum(FBS) (Sigma Chemical Co., St. Louis, Mo,U.S.A), Sodium bicarbonate (Sigma Chemical Co., St. Louis, Mo, U.S.A), and Penicillin /Streptomycin (Sigma Chemical Co., St. Louis, Mo, U.S.A.)

**Assay Method for Cytotoxic Activity**

The sensitivity of the human tumor cell lines to thymoquinone was determined by the Sulphorhodamine-B (SRB) assay. This method was carried out according to that of Skehan et al. (1990)[22]. HCT116 and HepG2 7 cell lines were plated in 96-multwell plates (5x10⁴- 105 cells/well in a fresh media) for 24 hrs before treatment with the tested sample to allow attachment of cells to the wall of the plate. Then, 200µl aliquot of serial dilution of chloroform extract (5.0, 12.5, 25, 50 µg/ml) was added then plates were incubated for 24, 48 and 72 hrs at 37°C. Control cells were treated with vehicle alone. Following 24, 48 and 72 hrs treatment; the cells were fixed with cold 50% trichloroacetic acid for 1 hr at 40°C. Four wells were prepared for each individual dose. Following 24, 48 and 72 hrs treatment, cells were fixed, washed and stained with Sulphorhodamine B stain (sigma, USA). Optical density was measured in an ELISA reader spectrophotometer (Tecan Group Ltd.-Sunrise, Germany). The percentage of cell survival was calculated as follows:


**RESULT AND DISCUSSIONS**

The coumarin compounds, Bergapten (1), Psoralene (2), Umbelone (3) gave (Greyish brown; Brownish orange and colorless) color with iodine-KI reagent respectively. The triterpenes, B- amyrin and Friedelane gave positive LB test for triterpenes and the sterol compounds gave positive LB test for sterols. Identification of the isolated coumarins, sterols and triterpenes was done by direct comparisons with authentic samples including Co-TLC spots and mixed m.p. Further confirmations of the structures of the isolated compounds were carried out by MS and 'H/NMR spectra and compared with the reported data [23] (Nielsen, 1971).
Separation and identification of lipoidal matters

Separation and investigation of unsaponifiable Fraction

GLC analysis of the unsaponifiable fraction proved to be a mixture of hydrocarbons, sterols and triterpenes. Identification of the compounds was carried out by comparison of their retention time with the available reference compounds (Table 1).

Separation and identification of Fatty Acid Fraction

GLC of the fatty acid methyl esters resulted in the identification of 9 fatty acids in which Palmitic acid C<sub>16</sub>:0 is the main saturated acids (13.86%) and Erusic acid C<sub>22</sub>:1 is the main unsaturated acids (19.84%) in Zilla spinosa plant. The data are presented in table (2).

Biological Activity

Anti –inflammatory activity

Z.spinosa has anti-inflammatory activity especially after 2 and 3 h compared with the control and that obtained by indomethacin (table 3); it significantly decreased the edema rate in comparison to the control group. The effect appeared at 2 h higher than that after 3 h. The anti-inflammatory activity of the plant could be due to the presence of triterpenes or phenolic compounds.

Analgesic activity

The plant extract has analgesic activity compared with the control and acetylsalicylic acid (table 4). Small dose of CHCl<sub>3</sub> extract produced 74.48 % protection against writhing induced by acetic acid while high dose produced 77% protection compared to 90.41% protection produced by acetylsalicylic acid. Analgesic activity of the plant could due to the presence of phenolic compounds.
Table 2: GLC of Fatty Acid Methyl Esters of the aerial parts of Zilla spinosa

<table>
<thead>
<tr>
<th>Identified compound</th>
<th>No. of carbon atoms</th>
<th>R.R.T</th>
<th>Area %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic acid</td>
<td>16:0</td>
<td>0.6</td>
<td>13.86631</td>
</tr>
<tr>
<td>Palmitoleic acid</td>
<td>16:1</td>
<td>0.63</td>
<td>7.47101</td>
</tr>
<tr>
<td>Unidentified</td>
<td></td>
<td>0.64</td>
<td>4.18023</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>18:0</td>
<td>0.66</td>
<td>8.13365</td>
</tr>
<tr>
<td>Unidentified</td>
<td></td>
<td>0.68</td>
<td>5.36235</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>18:1</td>
<td>0.69</td>
<td>7.57919</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>18:2</td>
<td>0.71</td>
<td>4.30700</td>
</tr>
<tr>
<td>Linolenic acid</td>
<td>18:3</td>
<td>0.72</td>
<td>3.80876</td>
</tr>
<tr>
<td>Eicosanoic acid</td>
<td>20:1</td>
<td>0.8</td>
<td>5.91050</td>
</tr>
<tr>
<td>Unidentified</td>
<td></td>
<td>0.81</td>
<td>3.91527</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>20:0</td>
<td>0.83</td>
<td>5.15270</td>
</tr>
<tr>
<td>Erusic acid</td>
<td>22:1</td>
<td>1</td>
<td>19.94048</td>
</tr>
</tbody>
</table>

R.R.T = Relative Retention Time.

Table 3: Anti-inflammatorv effect of chloroform extract of zilla spinosa:

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg b.wt.)</th>
<th>1 hour</th>
<th>2 hours</th>
<th>3 hours</th>
<th>Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>28.65±2.65</td>
<td>40.12±4.12</td>
<td>31.87±3.09</td>
<td>27.06±2.61</td>
</tr>
<tr>
<td>Aqueous extract</td>
<td>200</td>
<td>28.68±2.47</td>
<td>34.31±2.78</td>
<td>36.33±2.42</td>
<td>30.19±2.22</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>22.97±2.75</td>
<td>33.53±1.94</td>
<td>29.86±2.96</td>
<td>32.09±3.14</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>10</td>
<td>19.56±0.38</td>
<td>25.77±2.33</td>
<td>15.92±1.41**</td>
<td>15.52±1.51**</td>
</tr>
</tbody>
</table>

Values represent the mean ± S.E. of six animals for each groups.

P<0.001 : Statistically significant from control using one way ANOVA. (Dunnett's test).

Anti-microbial effect

Z. spinosa has a weak antibacterial activity comprised especially of (G+), and a (G-) bacteria and negative Antifungal effect (Table 5).

The chloroform extract was tested for cytotoxicity to two human cancer cell lines, namely, colon (HCT) and liver (HEPG2) cell lines. The activities were expressed by the IC50 value and their results are shown in (Fig. 1 and 2). According to the American National Cancer Institute guidelines) Suffness and Pezzuto, 1990 extracts with IC50 values <30 μg/ml were considered active. It was found that the chloroform extract was active against colon (HCT116) and liver (HEPG2) human cancer cell lines with IC50, 14.5 and 15.7 μg/ml respectively. The cytotoxicity of chloroform extract gave a significant result in case of colon (HCT116).

Table 5: Comparison of Zone of Inhibition between standared antibacterial drug and chloroform extract of Zillaspinosa plant.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Inhibition zone diameter (mm/mg Sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Escherichia coli (G-)</td>
</tr>
<tr>
<td>Solvent: DMSO</td>
<td>0.0</td>
</tr>
<tr>
<td>Tetracycline (Antibacterial agent)</td>
<td>33</td>
</tr>
<tr>
<td>Amphotericin B (Antifungal agent)</td>
<td>15</td>
</tr>
<tr>
<td>Drug(crud extract)</td>
<td>15</td>
</tr>
</tbody>
</table>

Cytotoxicity Activity
CONCLUSION

*Z.* spinosa is rich in chemical constituents which could be used as a treatment agent. The plant extract also showed a promising biological activity which could be attributed to the presence of triterpene, saponins or phenolic compounds. So, checking out of the biological activities and the active agents from medicinal plants reported in traditional medicinal is of interest as they may also act as templates for drug derivatization.

REFERENCES