Chemical Constituents of *Colocasia esculenta* leaves extract in Relation to its Self Defense against the Cotton leafworm, *Spodoptera littoralis* (Boisd.)

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ABSTRACT

Plants evolved elegant and complex chemical systems to defend themselves against the pathogens and herbivores that attempt to consume them. Taro, *Colocasia esculenta* plant has self defense property; it may be containing natural organic substances, allelochemicals, which are biologically active. The anti-feeding and insecticidal activities of petroleum ether extract of *Colocasia esculenta* leaves were studied against the 4th instar larvae of *Spodoptera littoralis* under laboratory conditions. Anti-feeding activity was assessed based on anti-feeding indices under choice and no-choice conditions. The highest anti-feeding activity is reached under no-choice conditions and anti-feeding indices were concentrations dependent; also it was found that the extract exhibits larval toxicity, the toxicity of the extract increased with time post treatment. Petroleum ether extract of *Colocasia esculenta* leaves was subjected to saponification reaction; from the un-saponifiable matter of petroleum ether extract, two phytosterols; namely β-sitosterol and stigmasterol were isolated by column chromatography (CC) and thin layer chromatography (TLC). Their structures were elucidated by 1H-NMR and MS techniques. The presence of these two compounds in *C. esculenta* leaves may be one reason of its self defense. This is the first record on the isolation and identification of chemical constituents from *Colocasia esculenta* leaves.  

Key words: *Colocasia esculenta*, allelochemicals, Anti-feeding activity, insecticidal activity, saponification, phytosterol, *Spodoptera littoralis*.

INTRODUCTION

Plants are important sources of natural products with the potential to be developed as commercial active ingredients for pest control, where it is believed that most of the plants that remain un-attacked by insects probably contain anti-feeding compounds (Crombie, 1999; Pavela and Herda, 2007; Rani and Murty, 2009).

Taro, *Colocasia esculenta* (L.), is a member of Araceae family; it is a perennial herb with large leaves which resemble an elephant’s ear and bearing one or more underground stems. *Colocasia esculenta* plant has self defense property, where it is not be attacked by insects, it may be containing natural organic substances, allelochemicals, which are biologically active. Therefore, its extracts could be applied as natural pesticides, especially insecticides.

Anti-feeding chemicals play a major role in the unsuitability of non-host plants as food for insects, so they have been proposed as an alternative to synthetic insecticides (Belles et al., 1985). It is believed that most of the plants that remain un-attacked by insects probably contain anti-feeding compounds.

Anti-feedants are chemicals that have anti-feeding properties at low concentration, and they act on very specific sensory cells (anti-feedant receptors) in the pest. The neurons associated with these antifeedant receptors either prevent insect feeding (feeding deterrent effect) or cause cessation or slowing of further feeding (feeding suppressant effect), (Chapman, 1974).

Another mode of action of some anti-feedants is through an apparent ability block the function of a herbivore’s feeding-stimulant receptors, or an ability to bind directly to its normal feeding cues, such as sugars and amino acids (Mordue and Blackwell, 1993).

During the last two decades many attempts have been made to isolate and identify various naturally occurring biologically active compounds possessing insecticidal properties (Schoonhoven et al., 1998; Isman, 2006 and Dayan et al., 2009), among the main advantages reported by the use of natural products from plant origin, their narrow toxicity spectrum and less environmental impacts stand out (Jermy, 1990; Morimoto and Komal, 2000; Harborne, 2001).

The Egyptian cotton leafworm, *Spodoptera littoralis* (Lepidoptera: Noctuidae) is considered as one of the most injurious and widespread pest of different crops in Egyptian agriculture. This phytophagous pest attacks cotton and large number of other field crops and vegetables, ornamental plants and weeds in Egypt as well as Mediterranean and Middle East countries causing heavy damage in different parts of the host plants (Bayoumi et al., 1998 and El-Aswad et al., 2003).

The purpose of this study is to investigate the anti-feeding and insecticidal activity of petroleum ether extract of *C. esculenta* leaves against the cotton leafworm, *Spodoptera littoralis*. Isolation and purification of some bioactive organic plant constituents using column chromatography and thin layer chromatography.

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Structure elucidation and characterization of the isolated products using different spectral methods such as $^1$H-NMR and MS techniques.

MATERIALS AND METHODS

Plant material
Fresh green leaves were collected, cleaned and dried under room temperature. The dried leaves were homogenized to fine powder by using electric machine. The leaves powder packed in paper bags and stored in air tight container until use.

Extraction and Fractionation
Leaves powder (700 g) was extracted by continuous hot extraction process using soxhelt apparatus with methanol (MeOH), after extraction the resulting extract was evaporated to its 1/3 volume under vacuum at 40°C by using rotary evaporator to get the crude extract as a dark green residue.

Methanolic crude extract was fractionated by dissolving in a mixture of MeOH / H$_2$O (1:1), transferred in a separator funnel and re-extracted by petroleum ether (40-60°C). The extract was dried over anhydrous sodium sulphate and concentrated under vacuum, then kept in a refrigerator for further investigation.

Instrumentation
NMR: $^1$H-NMR were recorded at 300 MHz (at Cairo University, Nuclear Magnetic Resonance Laboratory), chemical shifts are given in δ (ppm) relative to TMS as internal standard material. MS Mass Spectra were recorded on a Ssimadzu GC/MS UB 1000 EX instrument (at Cairo University, Micro Analytical Center). Normal Column Chromatography CC was performed on silica gel Merck grain size 0.2-0.063 mm; TLC Thin Layer Chromatography performed on silica gel Merck GF 254 pre-coated plates 20 x 20 cm on aluminum sheets and polyamide plates (0.25 mm, Merk).

Tested insect
Spodoptera littoralis were obtained from laboratory susceptible strain which was not exposed to insecticidal contamination and maintained for more than 10 generations under laboratory conditions was obtained as egg masses from Plant Protection Research Institute, Dokki, Giza. The colony and experimental procedures were carried out under room conditions of 25±2°C, 75±5% R.H and 16:8 (L: D) light: dark.

Preparation of the tested concentrations
Considering the petroleum ether crude extract as 100%, a known weight of the crude was added to a least volume of the solvent to obtain stock solution as a readily made. Stock solution of tested extract was made prior to use. For more homogenization of the first dilution, 0.3% of triton x-100 was added to the diluted solution. Successive dilutions were carried out for the later using water to obtain serial of the tested concentrations.

Anti-feeding activity
Each experimental unit consisted of plastic Petri dish (9 cm diameter). Damp filter paper was placed on the bottom of each dish to avoid early drying of the leaf discs.

Fresh castor leaf (Ricinus communis L.) discs of 4 cm diameter were punched using cork borer. Every disc treated with pet. ether extract or the corresponding carrier solvent, once the solvent was evaporated; two starved larvae were deposited using forceps into the center of each disc and allowed to feed.

The consumption of leaf discs were expressed as dry weight (mg). The remaining of leaf discs were collected, dried at 40°C and weighed. For estimation the initial dry weight at the beginning of each bioassay; 5 groups of 8 leaf discs were dried and weight (Nelson et al., 2009; Pavela and Vrchotová, 2013). Anti-feeding activity of tested extract was assayed by using choice and no-choice test as follows:

Discs were dipped in 100 µl of 0.125, 0.25, 0.5 and 1% concentrations of the extract individually. Discs to which only the same amount of carrier solvent had been applied were used as control. In choice test; only alternating leaf discs were treated with the test concentration (C= control leaf disc; T= treated leaf disc), whereas in the no-choice test; all leaf discs were treated with the test concentration (T= treated leaf disc), a control variant (Cv= control leaf disc), with only the carrier solvent was established in every bioassay as a reference to stop the experiment. When approximately 50% of the control leaf discs (C) in choice test, and 75% (Cv) in no-choice was been eaten. Larvae were removed from the petri dishes. Five replicates were maintained for each treatment in every test (Nelson et al., 2009; Pavela and Vrchotová, 2013).

From choice test data; it could be calculated:

Feeding deterrence index (FDI) = 100 [(C-T) / (C+T)], where C and T are the control and treated leaf weights consumed by the insect (Sadek, 2003; Pavela, 2010) This index evaluates the potential of a substance to induce the cessation on feeding when tasted by an insect, and whether continues feeding in an alternative source of food, due to the activation of anti-feeding receptors.

From combined data of no-choice test and the control used to stop the experiment, it could be calculated: Feeding inhibition index (FII) = 100 [(Cv - T) / Cv], this index evaluates the potential of a substance to inhibit the feeding when there is no-alternative source of food. It helps to evaluate the potential feeding inhibition that might be produced in no-treated foliar discs as a consequence of previous feeding in treated discs, that gives an idea of possible toxicity effects (Bentley et al., 1984).
Insecticidal activity

Laboratory experiments were conducted to study the toxic activity of pet. ether extract against the 4th instar larvae of *S. littoralis*, the experiments were carried out using leaf dipping technique as described by Sadek (2003). The larvae were allowed to feed on treated leaves only for 48 h, then on untreated leaves till death or pupation occurred. Three replicates of 10 larvae/each were used for each concentration in addition to control. Mortality of larvae was recorded daily and accumulative larval mortality was calculated till pupation and corrected by Abbott’s formula (1925).

Statistical analysis

Data were subjected to statistical analysis to evaluate efficiency of tested extract against the 4th instar larvae of *S. littoralis*, mortality percentages were corrected according to Abbott’s formula and then subjected to probit analysis. The toxicity lines were drawn on log concentration-probit paper and statistically analyzed according to Finney’s method (1997). The LC$_{50}$, LC$_{90}$ and slope values and their 95% confidence limits of tested extract were also estimated.

**Abbott’s formula:**
Corrected larval mortality percentage =

$$T - C / 100 - C$$

Where; T: Larval mortality percentage in treatment
C: Larval mortality percentage in control.

Separation of petroleum ether fraction

The pet. ether fraction was evaporated to its 1/3 volume, where a dark green residue was obtained. The residue was subjected to saponification reaction; hydrolysis with 10% alcoholic sodium hydroxide over water bath under reflux for 30 min., cooling, then diluted with water and extracted with diethyl ether afforded the unsaponifiable fraction.

The unsaponifiable matter was subjected to column chromatography using silica gel as adsorbent. Elution was carried out using pet. ether, followed by gradual addition of methylene chloride in increasing amount, till reaching methylene chloride 100%.

The sub-fractions (100 ml each) were collected and combined after monitoring by TLC on silica gel plates using toluene: acetone (90: 10) as solvent system. The spots were detected using UV (254 and 366 nm) before and after spraying with sulphorpic acid reagent and heating in oven at 110º C. The sub-fractions (8-12) were reacted positively with sulphoric acid reagent. These fractions were eluted using pet. ether: ethyl acetate (80: 20) as solvent system and subjected to TLC, It afforded a mixture of two compounds I and II of R$_f$ = 0.42, which gave deep violet colour on spraying with p-anisaldehyde-sulphoric acid reagent. It yielded a colourless crystals on evaporation to dryness.

RESULTS

Anti-feeding activity

Anti-feeding activity of pet. ether extract of *C. esculenta* leaves was assessed based on anti-feeding indices, where higher anti-feeding index normally indicated decreasing rate of feeding. It was observed that the anti-feeding activity varied depending on concentrations. The highest anti-feeding activity was under no-choice conditions; where no-choice test seems to be more sensitive than choice test, it was observed that the anti-feeding activity increased as the concentration of extract increased. Based on FII values, it was found that anti-feeding activity of petroleum ether extract at conc. 0.125 & 0.25 & 0.5 & 1% in no-choice test were 25.96 & 49.62 & 60.62 & 79.30 %. While, based on FDI values, the anti-feeding activity of petroleum ether extract at conc. 0.125 & 0.25 & 0.5 & 1% in choice test were 13.60 & 26.26 & 35.95 & 58.83%, respectively. (Table 1).

<table>
<thead>
<tr>
<th>Extract</th>
<th>Concentration %</th>
<th>Choice FDI %</th>
<th>No-choice FII %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petroleum ether</td>
<td>0.125</td>
<td>13.60</td>
<td>25.96</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>26.26</td>
<td>49.62</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>35.95</td>
<td>60.62</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>58.83</td>
<td>79.30</td>
</tr>
</tbody>
</table>

Table (1): Anti-feeding activity of pet. ether extract of *Colocasia esculenta* leaves against the 4th instar larvae of *S. littoralis* after choice and no-choice test.

In the choice test Feeding deterrence index (FDI) = 100[(C-T) / (C+T)], where C and T are the control and treated leaf weight consumed by the insect. In the no-choice test, Feeding inhibition index (FII) = 100[(Cv-T) / Cv], (T= treated leaf disc), a control variant (Cv=control leaf disc), with only the carrier solvent was established in every bioassay as a reference to stop the experiment.

Insecticidal activity

The toxic action as initial lethal effect of investigated extract to the 4th instar larvae of *S. littoralis* was evaluated under lab-conditions. The main criteria of the toxicity regression lines; LC$_{50}$, LC$_{90}$, and slope values were used as parameters in evaluation the insecticidal activity of tested extract. The insecticidal activity of petroleum ether extract was investigated by feeding of newly molted 4th instar larvae for 48 hrs on castor bean leaves treated with 0.125, 0.25, 0.5 and 1% of the extract followed by feeding on untreated leaves till pupation under laboratory conditions.
It was observed that the toxicity increased with time post treatment, when pupation occurred serious abnormalities were observed, where ec dysis and sclerotization were incomplete. (Table 2).

### Table (2): Insecticidal activity of pet. ether extract of C. esculenta leaves against the 4th instar larvae of S. littoralis

<table>
<thead>
<tr>
<th>Extract</th>
<th>LC₅₀ (%) and confidence limits</th>
<th>Slope</th>
<th>LC₅₀ (%) and confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petroleum ether</td>
<td>0.029 (0.001 – 0.075)</td>
<td>0.718 ± 0.213</td>
<td>1.784 (0.861 – 21.352)</td>
</tr>
</tbody>
</table>

The extract showed insecticidal activity against the 4th instar larvae of S. littoralis, so the extract will be studied in details. Two compounds I and II were isolated from un-saponifiable fraction by using thin layer chromatography as colourless crystals, Rᵣ = 0.42. They gave deep violet colour upon spraying with p-anisaldehyde-sulphuric acid reagent indicating it's a steroidal or triterpenoidal nature.

The ¹H-NMR spectrum of compounds I and II, δH 0.83 (3H, d, Me-26), δH 0.81 (3H, d, Me-27) and δH 0.85 (3H, t).

The ¹H-NMR spectrum revealed the presence of six methyl groups in the up field region showing that the compound is aliphatic and may be steroidal or triterpenoidal compound. The spectrum indicating the presence of a multiplet at δH 3.52 ppm for one proton H-3 indicating its steroid nature. An olefinic proton appeared as broad singlet at δH 5.34 ppm (distorted doublet (br s), which was assigned for H-6 suggesting the presence of a Δ²⁻ 3β- hydroxy-sterol. The spectrum indicated the presence of two tertiary methyl proton signals at δH 0.691 and δH 1.01 ppm, corresponding to (Me-18, Me-19), respectively. The side chain signals appeared at δH 0.92 (3H, d, Me-21) Me-29 and δH 1.87 (1H, m, H-25), suggesting that the sterol has a Stigmast-5-en-3β-ol skeleton. Based on all spectral data, compound I is β-sitosterol, (Table 3).

The mass spectral analysis of compound I showed the presence of ion peak M⁺ at m/z 414 (82%) corresponding to the molecular formula C₂₅H₄₀O. The spectrum also showed an ion peak at m/z 396 (54%) due to expulsion of water molecule. Another ion peak at m/z 381 (40%) due to further expulsion of methyl group, while the fragment peak at m/z 329 (77%) is due to the formula C₂₁H₃₂O, 303 (73%) is due to C₂₁H₃₅O and the fragment peak at m/z 231 (47%) is due to C₁₆H₂₃O. In addition, an ion peak at m/z 273 (45%) due to expulsion of the side chain C₁₀H₂₁ from M⁺ ion, ion peak at m/z 255 (60%) due to further expulsion of one molecule of H₂O and the ion peak 213 (91%) is due to the formula C₁₆H₂₃. The fragmentation pattern was in agreement with the reported spectrum in WILEY and NIST libraries that obtained by GC/ MS; identified for β-sitosterol.

### Table (3): ¹H-NMR of compounds I and II recorded in CDCl₃

<table>
<thead>
<tr>
<th>H atom</th>
<th>δ value (ppm), multiplicity</th>
<th>δ value (ppm), multiplicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P-I</td>
<td>P-II</td>
</tr>
<tr>
<td>3</td>
<td>3.52 (1H, m)</td>
<td>3.52 (1H, m)</td>
</tr>
<tr>
<td>6</td>
<td>5.34 (1H, br s)</td>
<td>5.34 (1H, br s)</td>
</tr>
<tr>
<td>Me-18</td>
<td>0.69 (3H, s)</td>
<td>0.69 (3H, s)</td>
</tr>
<tr>
<td>Me-19</td>
<td>1.01 (3H, s)</td>
<td>1.01 (3H, s)</td>
</tr>
<tr>
<td>20</td>
<td>1.48 (1H, m)</td>
<td>1.48 (1H, m)</td>
</tr>
<tr>
<td>Me-21</td>
<td>0.92 (3H, d)</td>
<td>0.92 (3H, d)</td>
</tr>
<tr>
<td>22</td>
<td>1.01 (2H, m)</td>
<td>4.98 (1H, dd)</td>
</tr>
<tr>
<td>23</td>
<td>1.26 (2H, m)</td>
<td>5.12 (1H, dd)</td>
</tr>
<tr>
<td>25</td>
<td>1.87 (1H, m)</td>
<td>1.87 (1H, m)</td>
</tr>
<tr>
<td>Me-26</td>
<td>0.83 (3H, d)</td>
<td>0.83 (3H, d)</td>
</tr>
<tr>
<td>Me-27</td>
<td>0.81 (3H, d)</td>
<td>0.81 (3H, d)</td>
</tr>
<tr>
<td>Me-29</td>
<td>0.85 (3H, t)</td>
<td>0.85 (3H, t)</td>
</tr>
</tbody>
</table>

The mass spectral analysis of compound II showed M⁺ at m/z 412 (47%) corresponding to the molecular formula.
C_{20}H_{18}O. The spectrum also showed an ion peak at m/z 394 (10%) due to expulsion of water molecule from M+, ion peak at m/z 379 (9%) due to further expulsion of methyl group and ion peak at m/z 351 (22%) due to further expulsion of C_3H_6 group. The fragment peaks at m/z 255 (64%) is due to the formula C_{19}H_{37}, at m/z 213 (36%) due to the formula C_{18}H_{21} and the ion peak at m/z 199 (20%) is due to C_{18}H_{19}, the base peak at m/z 55 (100%) is due to the formula C_3H_2. In addition, an ion peak at m/z 171 (33%) is due to the formula C_{13}H_{15}, and at m/z 159 (68%) is due to C_{12}H_{13} and 145 (61%) corresponding to the formula C_{11}H_{15}. the fragmentation pattern was in agreement with the reported spectrum in WILEY and NIST libraries that obtained by GC/ MS; identified for stigmasterol.

Based on all spectral data the compound I is stigmast-5-en-3β-ol which is known as β- sitosterol. The compound II is stigmas-5, 22-dien-3-ol which is known as stigmasterol.

DISCUSSION

In lepidopteran larvae, terpenoids and steroids block the stimulatory effects of glucose, sacarose and inositol on chemosensory receptors cells located of the insect mouth parts and they could also act on receptors in other ways (Greshenzon and Croteau, 1991) and (Greshenzon and Dudareva, 2007). It was found that steroids, terpenoids, phenols, coumarins have anti-feeding and growth inhibiting effects on S. littoralis larvae (Pavela, 2007).

The obtained results from insecticidal activity of pet. ether extract are parallel with anti-feeding activity where the extract recorded maximum FII index (94.39%) in no-choice at conc. 1%. These results are in agreement with Bentley et al., (1984); they showed that the calculated FII index from no-choice test is an indication of possible toxicity effect.

The 1H-NMR and mass spectra of compounds I and II revealed that the two compounds which were isolated and identified together from unsaponifiable fraction of pet. ether fraction are β-sitosterol (I) and Stigmasterol (II) which were isolated and characterized previously from the aerial parts of Ferulago bernardii by Khalighi et al., (2006). Also, Singh et al., (2013) isolated and characterized β-sitosterol and stigmasterol compounds together from the pet. ether extract of the aerial parts of Cassia species; C. renigera, C. pumila and C. nodosa.

Stigmasterol, the active constituent of Cacalia tangutica was found to be toxic to Spodoptera litura and its action was more marked in comparison to the other active constituents of the plant (Kaur et al., 2011)

This is the first record on the isolation and structure elucidation of chemical constituents from Colocasia esculenta leaves. The presence of these two compounds in C. esculenta plant may be one reason of its self defense.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Hamdy Abd El-Azeim Soliman; Chemistry Department, Faculty of Science, Suez Canal University; for his unlimited help and continuous support.

REFERENCES


المكونات الكيميائية لمستخلص أوراق نبات القلقاس وعلاقتها بخاصية الدفاع الذاتية ضد دودة ورق القطع

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الملخص العربي

تطلق النباتات مركبات كيميائية معقدة لتعفيها عن نفسها من أمراض وأفات التي تحاول مهاجمتها. يتميز نبات القلقاس بخاصية الدفاع الذاتية حيث أنه لا يصاب بالأطعمة والأمراض، و ذلك ربما لاحتوائه على مركبات عضوية طبيعية لها نشاط بيولوجي. لذلك تم دراسة تأثير مستخلص الأثير البترولى لأوراق نبات القلقاس كمانع للفكرية ودراسة تأثيره السام على إفراز العمر الرايع لدودة ورق القطع تحت الظروف المعملية. حيث دراسة تأثير منع الفكرية لمستخلص عن طريق الاختبار الاختياري ونواص مرسوم من الفكرية تتعمد على تركيز المستخلص. أظهرت النتائج أيضا ان المستخلص حقق سمية ضد اليرقات حيث تزداد هذه السمية بمرور الوقت بعد المعالجة. تم أجراء عملية التصبيح على مستخلص الأثير البترولى لأوراق نبات القلقاس وفصل الجزء غير المصبى، ثم الفصل والتعرف على مركبين استيرويدين (بيتا-سيتوستيرول) و (ستيماستيرول) عن طريق طرق الكروماتوجافى العمودى، كروماتوجرافى الطبقة الرقيقة، طيف الرنين النووى المغناطيسي للمهيدروجين و طيف الكتلة، وجود هذه المركبات في مستخلص الأثير البترولي ربما يكون من أحد أسباب حسبية الدفاع الذاتية لأوراق نبات القلقاس.