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Butterflies Extracts Show Antibacterial Activity

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Abstract

Extracts of several British butterfly species were tested and shown to possess powerful bactericidal activity against gram-positive bacteria (tested on *Staphylococcus aureus* and *Bacillus anthracis*). The active compounds in the grass-feeding species were identified as hydroxylated pyrrolizidine alkaloids (PAs) related to loline with nitrogen at C-1. Lolines are known insecticidal and insect-deterrent compounds that are produced in grasses infected by endophytic fungal symbionts. Lolines also increase resistance of endophyte-infected grasses to insect herbivores. The butterfly-isolated pyrrolizidine alkaloids appear to be novel and non-toxic to human cells such as HaCat human skin keratinocytes and Hep-2 human epithelial cells. The discovery of novel agents from butterflies could lead to the development of new antimicrobials.

Keywords

Pyrrolizidine Alkaloids, British Butterflies, *Staphylococcus aureus*, Antimicrobial

1. Introduction

Staphylococcus aureus are a Gram-positive aerobic bacteria that are usually non-pathogenic and are part of the normal skin flora. But once they produce toxins, they can cause major clinical infections and foodborne illnesses. These bacterium developed resistance to multiple antibiotics, and antibiotic resistance is on the rise globally. *S. aureus* cause diseases primarily by production of virulence factors such as enterotoxins and account for approximately 90,000 deaths a year in the US, resulting in high economic costs on patients and hospitals. Because

[#]deceased.

this resistance causes a serious public health problem, there is a great need for development of novel approaches to combat *S. aureus* infections [1] [2].

Bacillus anthracis are gram-positive, spore-forming bacterium that belongs to the *B. cereus* group, which includes *B. cereus, B. thuringiensis, B. mycoides, and B. weihenstephanensis* [3]. It is a bioweapon because of its tough, environmentally resistant endospore and its ability to cause lethal inhalational anthrax. It causes disease through the secretion of toxins, such as lethal toxin and edema toxin, and a capsule encoded by the genes on two plasmids [4] [5].

In our previous work, we showed that green tea extract and phytochemicals like epigallocatechin gallate (EGCG) that are found in green tea, have high bactericidal activity against Gram-positive bacteria [6]. In Gram-negative bacteria such as *Escherichia coli*, EGCG also was shown to damage bacterial membranes and degrade exopolysaccharides, resulting in the destruction of biofilms [7].

After a butterfly caterpillar hatches from an egg, the caterpillar feed on fresh leaves of flowering plants that contain secondary metabolites, which include a wide variety of phytochemicals. Ingestion of these phytochemicals can provide natural protection to the insect. In addition, the butterfly contains antimicrobial agents that are produced within the butterfly and are part of its own immunity [8]. In this study, we tested the effect of extracts of butterfly species on the Gram-positive bacteria *S. aureus* and *B. anthracis* and the Gram-negative bacteria *Pseudomonas aeruginosa* and *Proteus mirabilis*.

2. Materials and Methods

2.1. Sources of Butterflies and Grasses

The study focused on grass-feeding species of butterfly but also included nettle (Urtica dioica) and milkweed-feeding Monarch butterfly. The majority of Lepidoptera and recently hatched larvae used were collected in the UK, in the woodland and surrounding meadows of Ashton Wold. Various grasses, suitable as food plants for the grass-feeding butterflies, were placed in sterilized loam in wooden trays and monitored for endophytic fungi. Each tray was then covered with white muslin and watered daily. First instars caterpillars were placed on the grasses, after which their development and habits were studied and compared. These trays were situated and supervised in a greenhouse, in intermittent sunshine, at Ashton Wold with an average temperature of 600˚F. Larval food plants for different butterflies is shown in **Table 1**.

2.2. Bacterial Strains and Growth Conditions

S. aureus (strain ATCC 6538), *S. aureus* strain Smith diffuse [1], *B. anthracis* strain Sterne, a non-pathogenic vaccine strain [5], *E. coli* (USDA strain no. 16591), *P. aeruginosa* (strain ATCC 15442), and *Proteus mirabilis* (ATCC 29906) were grown in Luria-Bertani (LB) broth.

2.3. Antibacterial Activity Assay

S. aureus, B. anthracis, P. aeruginosa or *P. mirabilis* (1000 colony forming units

Table 1. Larval food plants for different butterflies.

Butterfly	Larval food plants
Peacock Red Admiral	Nettle (*Urtica dioica*) Thistle
Silver-washed Fritillary	Violets
Large Tortoiseshell	Young leaf tips of trees most commonly Elm (*Ulmus*) spp and sometimes Willows (*Salix*) spp and Polar (*Populus* spp.)
Cryptic larvae of: Meadow Brown, Ringlet, Marbled White, Gatekeeper. Skipper	Various grasses (Gramineae) Mountain Ringlet also feeds of Dock (*Rumex*)
Brimstone Orange Tip	Young leaves of Buckthorn (*Rhamnus* spp.) Lady's smock (*Cardamines pratensis*)
Monarch, when it reaches the UK	Milkweeds (*Asclepia* spp.)

(CFU)) were freshly prepared in 1 ml LB broth. Three µl of total extract or 5 µl of each HPLC-eluted fraction were added to 97 or 95 µl of bacteria respectively in sterile 96 well plates. Bacteria were grown for 24 hours at 37˚C and density determined at OD 650 nm. A sample was then also streaked on LB agar plates to confirm OD results. Control buffer was 3 µl methanol (to a final 3%) or HPLC carrier buffer (0.1 M NH_4OH). Minimal Bactericidal Concentration (MBC) of butterfly extracts was performed by adding 5 µl of material in question diluted in water or methanol to 1000 bacteria in culture media and growth continued overnight at 37˚C. Optical density was determined at 650 nm and a 3 µl sample was grown on LB agar plates and CFU counted. Of note is that no bacterial growth was observed below A650 = 0.15.

To test for the effect of loline and loline derivatives on bacterial growth, S. aureus and E. coli were grown as described above with or without 250 µg/ml of Loline, N-Acetylnorloline, N-Acetyl-5,6-dehydrololine, or N-Demethylloine (ChemFaces, Wuhan, Hubei, China). 0.5% DMSO was used as a negative control.

2.4. Treatment with Proteinase K

Total butterfly extracts (3 µl) were incubated for 30 min at 37˚C with 10 µg Proteinase K in 100 µl of water, or with water only as a control. Sample was then incubated at 65˚C for 10 min to inactivate Proteinase K. Treated samples were evaporated, resuspended in 3 µl methanol and tested for antibacterial activity as described above. To ensure that the Proteinase K was active, the same amount of enzyme (10 µg) was added to 100 µg BSA for 30 min at 37˚C, sample applied to SDS PAGE and gel stained by coomassie. The fact that BSA was degraded indicated that indeed the enzyme was active.

2.5. Electrospray Ionization Mass Spectrometry

Butterfly sample alkaloids were analyzed by GC-MS and by reaction with the classic alkaloid reagent Dragendorff's [9] both before and after a clean-up of

aqueous ethanol extracts on IR-120 (H+ form cation exchange resin). While lolines react with Dragendorff's reagent, the active compounds did not. Analysis of butterfly extracts and loline was carried out by GC-MS using a Perkin Elmer Autosystem XL gas chromatograph with a high polarity fused-silica column (Varian "FactorFour" VF-5ms column, 25 m × 0.25 mm i.d., 0.25 μm phase thickness). The carrier gas (helium) flow rate was 1 $ml \cdot min^{-1}$. Underivatised samples were run in 90% ethanol on a temperature program running from 50˚C (held for 3 minutes) to 300˚C over 30 minutes. Loline had a retention time of 12 minutes. Electron impact mass spectrometry of the column eluant was carried out using a Perkin Elmer TurboMass Gold mass spectrometer, with a quadrupole ion filter system, which was run at 250˚C constantly during analysis. The detector mass range was set to 45 to 350 amu. The temperature of the transfer line (GC to MS) was held at 250˚C. Samples were injected onto the column via a split vent (split ratio 50:1) through a fused silica narrow bore injection liner packed with deactivated quartz wool; the injection port temperature was maintained at 200˚C. The injection volume was 1 μl. System control, data collection and mass spectral analysis was carried out using Perkin Elmer TurboMass software (TurboMass v.4.4).

2.6. Effect on Human Cells

HaCat human skin keratinocytes or Hep-2 human epithelial cells were applied to Costar 96-well cell culture polystyrene plates (Corning Inc., Corning NY, USA) (10^4 cells/well) and grown to reach confluency at 37˚C in a 5% CO_2 humidified incubator in DMEM supplemented with 5% FCS. Increasing amounts of butterfly extracts (0 - 10 μl) were applied to the cells, cells incubated for 24 hrs, and cell viability determined colorimetrically by staining with 2,3-Bis(2-Methoxy-4-Nitro-5-Sulfophenyl)-5-(Phenylamino) Carbonyl]-2H-Tetrazolium Hydroxide (XTT) [10].

Human melanoma and ovarian cancer cells (SCC1) were incubated with increasing amounts of butterfly extracts (0 - 10 μl). Cells were plated out into 96 well tissue culture plates at an appropriate concentration and left overnight at 37˚C, 5% CO_2 and 100% humidity. The medium was replaced with 0.5 mg/ml of the Ringlet samples in complete medium and 10% v/v AlamarBlue. Controls included medium only and 100% distilled water. After 24 hours the medium was transferred into a 96 well plate and OD have been read at 560 nm and 600 nm. In the meantime, the cells were replenished with complete medium, without any drug, but with 10% v/v AlamarBlue. After 24 hours the OD was read again at 560 nm and 600 nm.

3. Results

3.1. Antibacterial Activity of Butterfly Extracts

Multiple species of butterflies (**Table 2**) were tested for the presence of defensive antibacterial chemicals. Initially, butterflies (Ringlet and Meadow Brown) were extracted by water or methanol and each extract tested for antibacterial activity against *S. aureus*. Because only the methanol-extracted material was active, further extractions from all other butterflies were carried out in methanol only. As

Table 2. Antibacterial activity found in various butterfly extracts against Gram-positive and Gram-negative bacteria. (+) growth (*i.e.* no antibacterial activity), (−) no growth (*i.e.* antibacterial activity).

	Growth of *Bacillus anthracis*	Growth of *Staphylococcus aureus*	Growth of *Proteus mirabilis*	Growth of *Pseudomonas aeruginosa*
Meadow Brown	−	+	+	+
Ringlet	−	+	+	+
Peacock	−	−	+	+
Monarch	−	−	+	+
Skipper	−	+	+	+
Gatekeeper Female	−	−	+	+
Gatekeeper Male	−	−	+	+
Marbled White female	−	−	+	+
Marbled White male	−	−	+	+
Large Tortoiseshell female	−	−	+	+
Red Admiral male	−	−	+	+
Brimstone male	−	−	+	+
Silver washed Fritillary female	−	−	+	+
Silver washed Fritillary male	−	−	+	+
Orange Tip male	−	−	+	+
Control 3% methanol	+	+	+	+

shown in Table 2, all extracts were active against gram-positive bacteria *B. anthracis* and most also against *S. aureus*.

None of the extracts were active against gram-negative bacteria *P. aeruginosa* and *P. mirabilis*. Antibacterial activity was retained after treatment of extracts with Proteinase K, suggesting that active components are not polypeptides.

MIC and MBC of each butterfly extract were tested and shown in Figure 1, and compared to those of conventional antibiotics. The MIC for *S. aureus* and *B. anthracis* e.g. of Red Admiral and Marbled White was 1:100, and Gatekeeper and Orange Tip was 1:10. The MBC was ten times higher than the MIC. MIC and MBC of ampicillin and ciprofloxacin were 0.1 μg/ml and 1.0 μg/ml respectively for these bacterial species, which is compatible with published data [11].

3.2. Toxicity Testing

To test if extracts are toxic to eukaryotic cells, HaCat human skin keratinocytes or Hep-2 human epithelial cells were incubated with increasing amounts of each

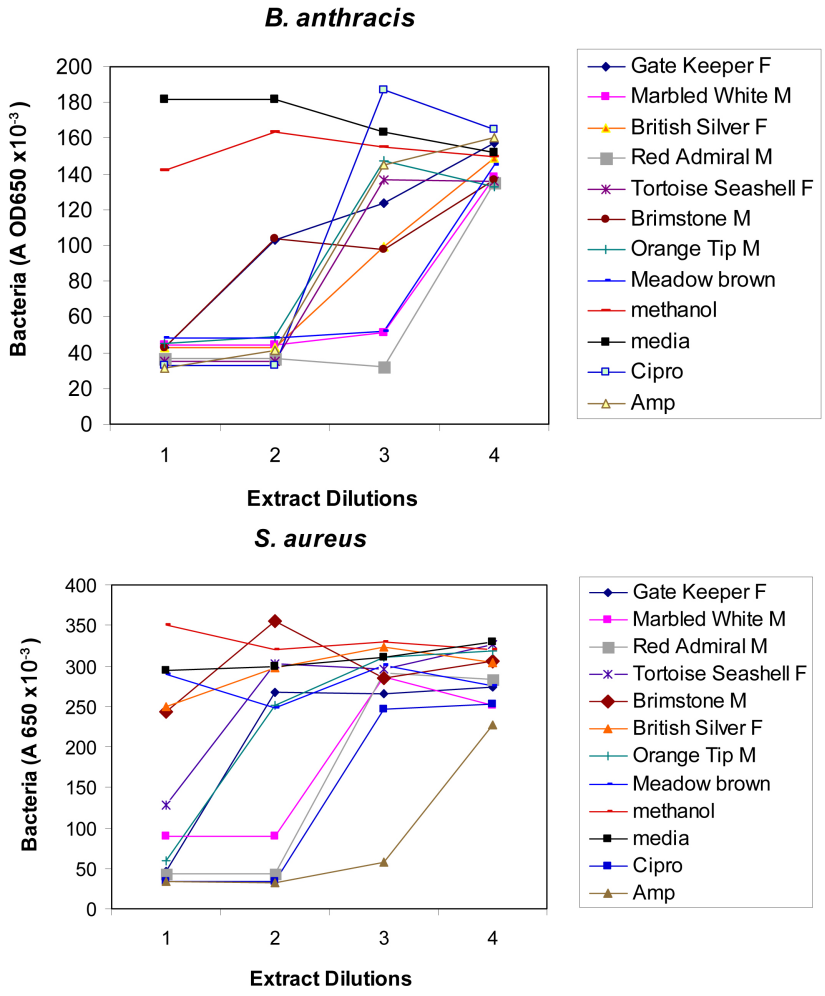

Figure 1. MIC test of butterfly extracts on growth of *S. aureus* and *B. anthracis*. Final dilutions of extracts: 1 = 1:10, 2 = 1:100, 3 = 1:1000, 4 = 1:10,000. Antibiotics final con-centration: Ciprofloxacin (Cipro, μg/ml) 1 = 1, 2 = 0.1, 3 = 0.01, 4 = 0.001. Ampicillin (Amp, μg/ml) 1 = 10, 2 = 1, 3 = 0.1, 4 = 0.01. Of note that Brimstone showed higher OD while almost no bacterial growth due to a certain opaqueness of the extract itself.

of the butterfly extracts, and 24 hrs later cell viability was tested colorimetrically using XTT [10]. Results (not shown) indicate that extracts had no visible signs of toxicity to the cells. Cytotoxicity was also tested on human melanoma and ovarian carcinoma cells by AlamarBlue, with no observable effect of any of the butterfly extracts.

3.3. Purification of Active Compounds

Crude methanol extracts were fractionated on a reverse phase HPLC column and eluted fractions were tested for antibacterial activity. As shown in Figure 2, most distinct active fraction of all extracts was the one eluted between 33% - 35% acetonitrile. Some additional active fractions were eluted around 46% - 49% acetonitrile but those were less distinctive and their activity was lost when re-fractionated using the same conditions.

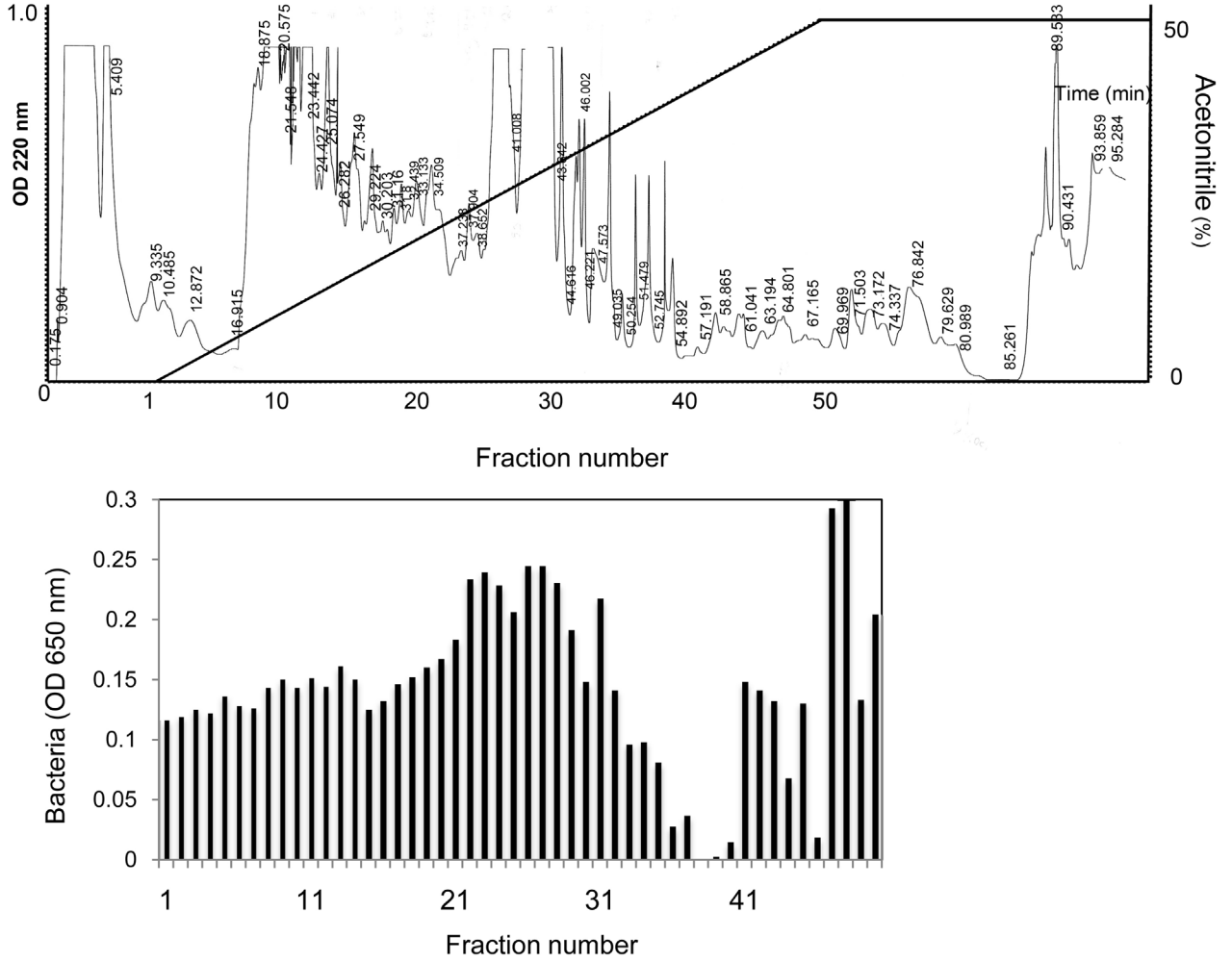

Figure 2. Sample HPLC purification and antibacterial activity profile of Meadow Brown extract. Top, HPLC profile determined at OD 220 nm. Bottom, antibacterial as-says of eluted fractions (bacterial growth determined at OD 650 nm).

3.4. HPLC Fractionation and GC-MS Analysis

The biologically active fractions from the HPLC separation of the butterfly extracts were analyzed by GC-MS and by reaction with the classic alkaloid detection reagent (Dragendorff), both before and after treatment of 70% or 90% aqueous ethanol extracts on cation exchange resin (**Figure 3**). Identification of loline-related compounds in the active fractions was based on comparison of the gas chromatographic retention times and characteristic mass spectra of the alkaloids found [12] [13]. The mass spectrum of loline gave a major ion at 82 amu (**Figure 4(a)**) and this ion is distinctive of loline derivatives (**Figure 4(b)** and **Figure 4(c)**). The compounds described here (**Figure 4(d)**), which are presumed to have the furan ring open, give distinctive mass spectra with major ions at either 84 or 86 (**Figure 3** and **Figure 4(d)**). The difference in the ions being either 84 or 86 is caused by opening of the furan ring to give an oxygen either on C-2 (giving major fragment 84 amu) or C-7 (major fragment 86 amu). Variations on these basic structures can occur such as unsaturation after opening of the furan ring and alkyl groups on the nitrogen at C-1. The active fractions were not reactive

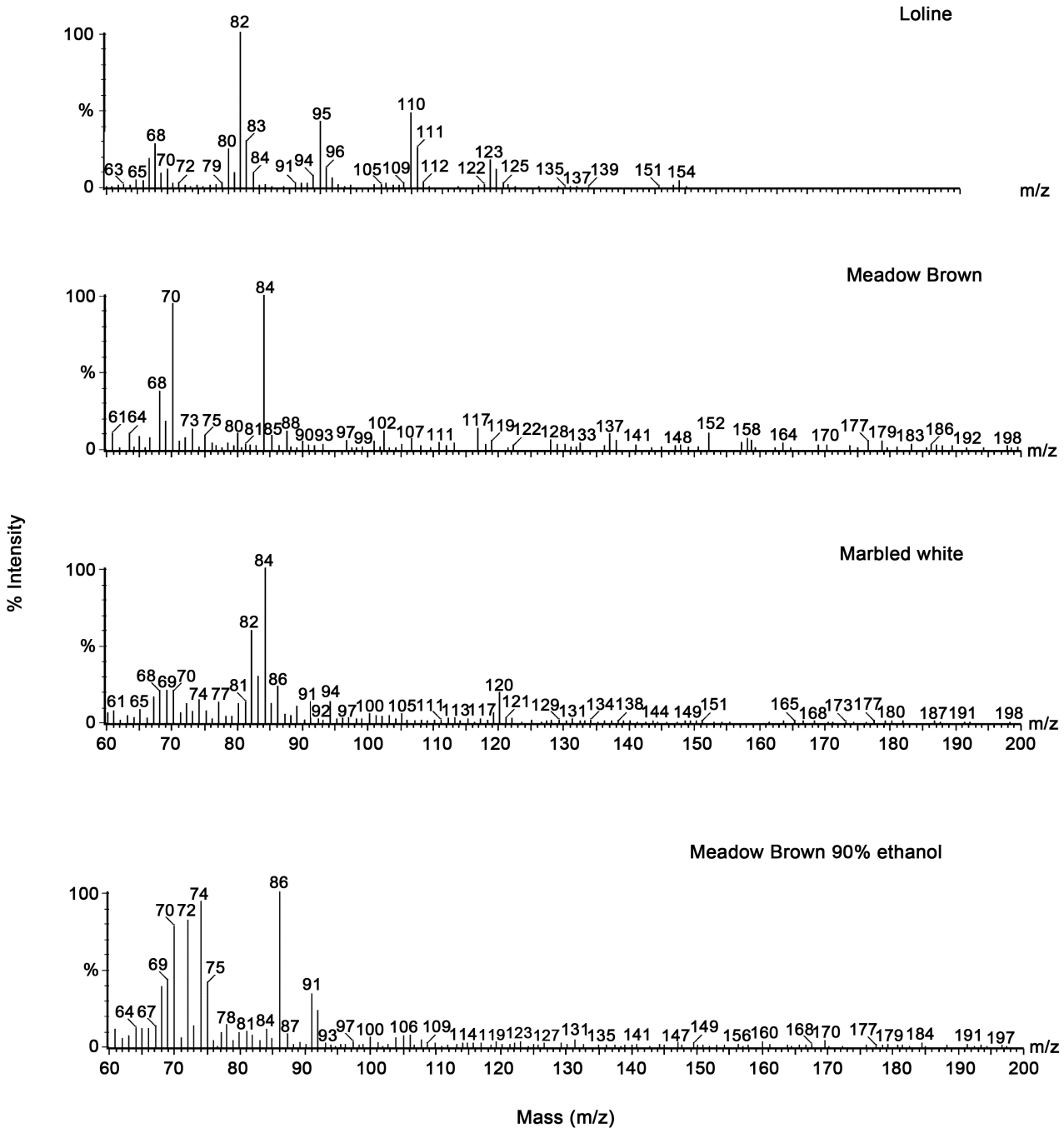

Figure 3. The mass spectrum of loline and characteristic mass spectra of loline-related compounds in butterflies from the fractions that have the antimicrobial activity.

with Dragendorff's reagent.

3.5. The Effect of Loline and Its Derivatives on Bacterial Growth

The effect of loline or its derivatives on gram positive (*S. aureus*) and gram negative (*E. coli*) bacteria was tested by growing the cells in the presence of 250 μg/ml Loline, N-Acetylorloline, N-Acetyl-5,6-dehydrololine, or N-Demethylloine. As shown in **Figure 5**, these molecules had no effect on bacterial growth,

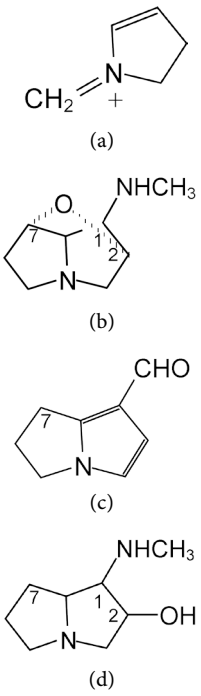

Figure 4. Chemical structures of (a) Loline major MS fragment at 82 amu; (b) Loline; (c) Danaidal; (d) The compounds described here which are presumed to have the furan ring open.

supporting our hypothesis that it is not loline itself that is causing the antibacterial effect observed in butterfly extracts.

4. Discussion

Our study describes the isolation of pyrrolizidine alkaloids (PAs) in grass-feeding British butterflies that are active against gram-positive bacteria (*S. aureus* and *B. anthracis*) but not against gram-negative bacteria (*P. aeruginosa* and *P. mirabilis*). Several of the grass food plants are known to contain modified or precursors of PAs [14]. Loline alkaloids are also produced by mutualistic fungi symbiotic with grasses, and may be the source of the PAs we have found in the adult butterflies [15] [16] [17] [18]. The association of the symbiotic fungal endophytes with the food plants of these species is widespread, but erratic. As shown in **Table 1**, the larvae of these butterflies feed on a variety of grasses, which include Perennial Rye-grass (*Lolium perenne*) and various *Fescues*, especially in the UK *Festuca rubra* and *Festuca ovata*. The Ringlet favours grass species such as Wood Millet (*Milium effusum*), Wood False-brome (*Brachypodium sylvaticum*), Cock's-foot (*Dactylis glomerata*), and sedges for example *Carex* spp., while the Meadow Brown prefers Bents (*Agrotis* spp.) and *Poa* spp. and in many cases, like the food plant of the Marbled White [16], the distribution of the eggs is also erratic. These grasses may possess the precursors of PAs, fungal parasites, symbionts, and various ecto- and [19] [20], but as far as is known, grasses do not themselves produce PAs. The occurrence of the endophytes can

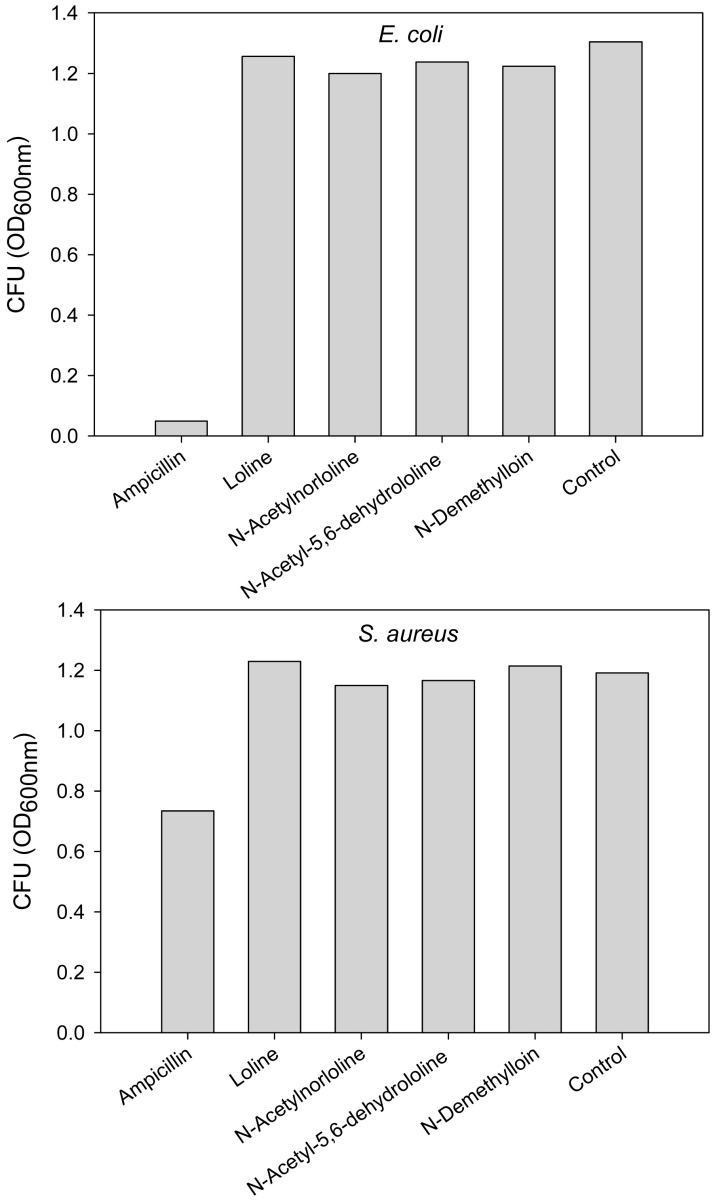

Figure 5. The effect of loline and its derivatives on bacterial growth.

vary from 100% to nil in the grass growing in meadows, on airfields, along road verges, in the roughs of golf courses and woodland rides etc. Richardson [20], has recorded over 200 grass endophytes). We therefore suggest that the saturated PAs that we found in the larva, pupa and adult of the semi-colonial Marbled Whites, were originally obtained from the symbiotic endophytes associating with their food [15] [16] [20]. In culturing the Mabled White larvae it was noted that they sought grass containing endophyte in preference to grass without the fungus.

The PA Danaidal (1-formyl-6,7-dihydro-5H-pyrrolizine) had been reported as a pheromone [21] in some exotic butterflies [22]. More recently classic PAs from plants such as Ragwort (*Senecio jacobaea*) have been reported to be anti-bacterial, however, they are too toxic to be suitable as oral drugs [23] [24] [25].

Interestingly, unsaturated PAs are one of the most widely spread plant poisons [26] [27] [28] and are probably the most poisonous single group of alkaloids. They are hepatotoxins converted to toxic pyrroles in vivo and cause death and liver damage [29]. In the United States, the less toxic saturated alkaloids are frequently used for the protection of agricultural crops, including lawns in gardens, from the persistent attack by a wide range of herbivorous insects. The PAs we discovered do not appear to be toxic to mammalian cells and are not the pro-toxic 1,2-dehydropyrrolizidines that form toxic pyrroles and so could potentially be used to derive a new family of drugs for microorganisms resistant to current antibiotics.

Loline, which is the best known of this group of PAs, or a few of its known derivatives tested, are not themselves antibacterial, but could form the basis for the development of a new class of antibiotics. Full synthesis of a range of alkaloids related to loline is underway to confirm the structure and function of the loline-related compounds present in the active fractions from the insect extracts.

The presence of a potential antibiotic in various British butterflies indicates an important area for research in both insects and the chemicals associated with their food plants. The results of our investigations emphasize the importance of the conservation of nature, not only its aesthetic value and interest, but also as an unquestionable benefit to humanity.

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