An extract from the frass of swallowtail butterfly (Papilio machaon) larvae inhibits HCT116 colon cancer cell proliferation but not other cancer cell types

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Abstract

Background

The frass of several herbivorous insect species has been utilised as natural medicines in Asia; however, the metabolite makeup and pharmaceutical activities of insect frass have yet to be investigated. Oligophagous Papilionidae insects utilise specific kinds of plants, and it has been suggested that the biochemicals from the plants may be metabolised by cytochrome P450 (CYP) in Papilionidae insects. In this study, we extracted the components of the frass of *Papilio machaon* larvae reared on *Angelica keiskei*, *Oenanthe javanica* or *Foeniculum vulgare* and examined the biological activity of each component. Then, we explored the expression of CYP genes in the midgut of *P. machaon* larvae and predicted the characteristics of their metabolic system.

Results

The components that were extracted using hexane, chloroform or methanol were biochemically different between larval frass and the host plants on which the larvae had fed. Furthermore, a fraction obtained from the chloroform extract from frass of *A. keiskei*-fed larvae specifically inhibited the cell proliferation of the human colon cancer cell line HCT116, whereas fractions obtained from the chloroform extracts of *O. javanica*- or *F. vulgare*-fed larval frass did not affect HCT116 cell viability. The metabolites from the chloroform extract from frass of *A. keiskei*-fed larvae prevented cell proliferation and induced apoptosis in HCT116 cells. Next, we examined the metabolic system in *A. keiskei*-fed larvae by RNA-seq analysis and found that the *A. keiskei*-fed larval midgut had different characteristics from the *O. javanica*- or *F. vulgare*-fed larval metabolic systems. We found that the CYP6B2 transcript was highly expressed in the *A. keiskei*-fed larval midgut.

Conclusions

These findings indicate that *P. machaon* metabolites might be useful as pharmaceutical agents against human colon cancer subtypes. Importantly, our findings show that it might be possible to use insect metabolic enzymes for the chemical structural conversion of plant-derived compounds with complex structures.

Background

Over one million species of insects have been recorded, and half of them are categorised as herbivorous insects that utilise plants [1, 2]. Frass from herbivorous insects has been used as a source of natural medicine in several countries. The reason for this is that frass contains insect-derived metabolites that may have enhanced biological activity compared with the biological activity of the metabolites the insects originally consumed from plants. Some medicinal herbs and frass from a stick insect, *Eurycnema*

versifasciata, are used to treat a variety of ailments, including diarrhoea, asthma, upset stomach and muscular pain, in Malaysia and China [3, 4]. In addition, *Bombyx mori* larval frass is thought to stimulate the adrenal gland and to decrease blood cholesterol content [5]. These examples demonstrate that the frass of several phytophagous insect species has been used as pharmaceutical agents in several countries to treat human diseases. The therapeutic effects of insect frass may depend on the insects' host plants, with the metabolites found in frass being converted into more effective metabolites than those found in the host plant tissues through the process of insect metabolism [6]. However, the process by which insect metabolism changes the biological activity of host plant metabolites is unclear.

Herbivorous insects are categorised into three types: (1) monophagous insects that utilise only one species of host plant, (2) oligophagous insects that utilise a narrow range of host plants, and (3) polyphagous insects that utilise a broad range of host plants [2]. Polyphagous insects have the advantage of being able to feed on a balanced diet at any time and in any place, while monophagous and oligophagous insects can specialise in utilising toxic metabolites from their host plants to protect themselves from predators. Heliconian butterflies, which are monophagous insects, produce cyanogenic glucoside, which is metabolised from their host plants in the Passifloraceae family [7]. Furthermore, troidine swallowtails, which belong to Papilionidae and feed on plants of the Aristolochiaceae family, sequester aristolochic acid from their host plants [8]. These monophagous and oligophagous insects are therefore difficult for predators to eat because they are unpalatable [9].

Cytochrome P450 (CYP), a drug-metabolising enzyme that is highly conserved in most organisms, has potential for biotechnological use because of its high diversity of catalysed reactions, and because high productivity is needed for such biotechnological applications [10, 11]. Most Papilionidae family members, including polyphagous and oligophagous insects, feed on furanocoumarin-containing plants [12]. Furthermore, oligophagous Papilionidae can metabolise furanocoumarins more efficiently than polyphagous insects by means of their CYPs [13, 14]. Thus, CYPs from oligophagous Papilionidae are a potential resource for biotechnology; however, their metabolic function in processing host plant metabolites, other than furanocoumarins, has yet to be investigated.

Larvae of *Papilio machaon*, which belongs to the Papilionidae family, are specialists of Apiaceae family plants, such as *Angelica keiskei*, *Oenanthe javanica* and *Foeniculum vulgare* (commonly known as fennel). These plants have been used as traditional medicine for a wide range of ailments, with their extracts showing various biological activities, including antitumour, anti-inflammatory, antioxidant and antiviral effects [15–17].

Therefore, we speculated that the metabolites of *P. machaon* larval frass, which includes host plant-derived metabolites, may contain biological activities of interest [18].

In this study, we aimed to evaluate the biological activity of the metabolites from the frass of *P. machaon* larvae fed on *A. keiskei*, *O. javanica* or *F. vulgare* using a human cancer cell line (HCT116), and we analysed the metabolic process of these metabolites in *P. machaon* larvae.

Results

Extracts from frass of P. machaon *larvae fed with* A. keiskei *specifically inhibit HCT116 cell viability*

First, we produced extracts from the frass of *P. machaon* larvae fed on *A. keiskei*, *O. javanica*, or *F. vulgare* using n-hexene, chloroform, and methanol (Fig. 1). Then, we compared the metabolic products from both *P. machaon* frass and the leaves of *A. keiskei*, *O. javanica*, or *F. vulgare* using thin-layer chromatography (TLC) plate chromatograms (Fig. 2, Supplementary Table 1). Staining the TLC plate chromatograms with sodium phosphomolybdate solution revealed two spots with calculated Relative to front (Rf) values ranging from 0.55 to 0.40 in the n-hexane extract (Hex. ext.) of frass from *O. javanica*-fed larvae (Fig. 2A lane 1, blue arrowheads), and we detected five spots with Rf values ranging from 0.55 to 0.31 in the Hex. ext. of frass from *A. keiskei*-fed larvae (Fig. 2A lane 3, orange arrowheads), and one spot with an Rf value of 0.36 in the Hex. ext. of frass from *F. vulgare*-fed larvae (Fig. 2A lane 5, grey arrowheads). Seven spots with Rf values from 0.61 to 0.13 were detected in the chloroform extract (Chl. ext.) of frass from *O. javanica*-fed larvae (Fig. 2B lane 1, blue arrowheads), and we detected five spots with Rf values ranging from 0.57 to 0.39 in the Chl. ext. of frass from *A. keiskei*-fed larvae (Fig. 2B lane 3, orange arrowheads), and five spots of Rf values from 0.59 to 0.18 in the Chl. ext. of frass from *F. vulgare*-fed larvae (Fig. 2B lane 5, grey arrowheads). Three spots with Rf values from 0.76 to 0.30 were detected in the methanol extract (Met. ext.) of frass from *O. javanica*-fed larvae (Fig. 2C lane 1, blue arrowheads), and we detected five spots with Rf values from 0.85 to 0.38 in the Met. ext. of frass from *A. keiskei*-fed larvae (Fig. 2C lane 3, orange arrowheads), and five spots of Rf values from 0.86 to 0.38 in the Met. ext. of frass from *F. vulgare*-fed larvae (Fig. 2C lane 5, grey arrowheads). We did not find these spots in the Hex., Chl. and Met. extracts of leaves from the *A. keiskei*, *O. javanica*, and *F. vulgare* plants (Fig. 2A,C, lane 2,4,6). In a previous study, the Chl. ext. of frass from *Poncirus trifoliata*-fed *Papilio xuthus* larvae inhibited cell viability of the human pancreatic cancer cell line MIA PaCa2 [19].

Thus, we chose the Chl. extracts produced from the frass of *P. machaon* larvae that had been fed on *A. keiskei*, *O. javanica* or *F. vulgare* for use in subsequent investigations. These extracts were separated using open column chromatography (Supplementary Fig. 1) to obtain an active fraction for use in testing the biological activity of the extracts against the human cancer cell line HCT116. Next, we evaluated the cell viability under exposure to these fractions using the HCT116 cell line by WST-1 or WST-8 assay. The Chl. ext. of frass from *P. machaon* larvae that fed on *O. javanica* was separated into three fractions, and these fractions did not decrease viability in the HCT116 cells (Fig. 3A and B). Similarly, the Chl. ext. of frass from *P. machaon* larvae that fed on *F. vulgare*, which was separated into four fractions, did not decrease viability in the HCT116 cells (Fig. 3C and D).

However, the Chl. ext. of frass from *P. machaon* larvae that fed on *A. keiskei* was separated into eleven fractions, and we found that Fraction (Fr.) 1 showed significantly decreased viability (10.4%) in HCT116 cells (Fig. 4A and B). Additionally, we measured proliferation of HCT116 cells by using the bromodeoxyuridine (BrdU) assay. We observed significantly decreased BrdU uptake in HCT116 cells treated with 5 μg/mL of Fr. 1 from the *A. keiskei*-fed larval frass (Fig. 4C).

Furthermore, we evaluated the cell death of Fr. 12 and Fr. 13, and found that cell death was induced in Fr. 12 but not in Fr.13 (Fig. 4-D). Subsequently, we evaluated the cell viability of Fr. 12 to several human cancer cell lines. Fr. 12 affected only the HCT116 cell line and did not significantly affect the viability of the A549, HeLa, HepG2, MIA PaCa2, and TGBC1TKB cancer cell lines. Furthermore, Fr. 12 did not affect the viability of HFSKF-II cells, which are a human normal fibroblast cell line (Fig. 4-E). Therefore, Fr. 12 only strongly affected the colon cancer cell line.

Examination of metabolites in larval food plants using the TUATinsecta database

Fr.12, which was extracted from the frass of *P. machaon* larvae that were fed *A. keiskei*, inhibited HCT116 cell viability, whereas the fractions from the frass of larvae that were fed *O. javanica* or *F. vulgare* had no effect on the HCT116 cell viability. Thus, we predicted that the chloroform extract frass from *A. keiskei*-fed larvae contained compounds that differ from compounds in the frass of larvae fed with *O. javanica* or *F. vulgare*. To examine the compounds of each food plant—*O. javanica*, *F. vulgare*, or *A. keiskei*—we utilised the TUAT-insecta database, which compiles data on the relationships for herbivorous insects, their host plants and related biological activity [20]. This interrogation revealed that the metabolites of *A. keiskei*, *O. javanica* and *F. vulgare* are mainly classified into 3 groups based on their chemical structures: phenolics, terpenes/steroids and alkaloids. Notably, chalcones, including phenolics and flavonoids, are found in only *A. keiskei* (Table 1).

Mechanism of action of metabolites from the frass of P. machaon *larvae fed with* A. keiskei *on HCT116 cells*

To investigate the mechanism by which Fr. 12 decreases HCT116 cell viability, we employed transcriptome analysis using total RNA of the HCT116 cells after 48 hours of treatment with 5 μg/mL of Fr. 12. Then, differentially expressed genes (DEGs) were identified based on the DEG analysis (false discovery rate, FDR < 0.05). Gene enrichment analysis showed that the DEGs were related to microtubule cytoskeleton organisation (GO: 0000226), a Gene Ontology (GO) term involved in cell division (Fig. 5). Additionally, we found that 19 genes out of the top 50 DEGs were those involved in HCT116 cell proliferation (15 genes), the cell cycle (6 genes) and apoptosis (9 genes) (Table 2). Therefore, our transcriptome analysis showed that Fr. 12 may induce inhibition of cell proliferation and apoptosis in HCT116 cells.

Exploration of metabolic enzymes that convert metabolites, including those from larval food plants, to active agents

Extracts from the frass of *P. machaon* larvae fed with *A. keiskei* inhibited HCT116 cell viability. This indicates that metabolic enzymes in *P. machaon* convert metabolites, including those from its food plants, to active agents. Thus, we compared metabolic enzymes among the larvae that were fed *A. keiskei*, *O. javanica* or *F. vulgare* by using midgut and fat body transcriptome analysis. Importantly, CYP

plays a critical role in the metabolism of plant secondary metabolites [41]. Therefore, we focused on CYP genes as metabolic enzymes that convert metabolites, including those of food plants, to active agents. Principal component analysis (PCA) was performed using R software to investigate the characteristics of the expressed genes, and these genes were categorised into four patterns (Fig. 6A). The CYP genes expressed in the midgut of *A. keiskei*-fed larvae differed significantly from those in the midgut of *O. javanica*- and *F. vulgare*-fed larvae (Fig. 6A). Next, we examined whether these CYP genes were upregulated, and we found that the expression of CYP6B2 genes was significantly upregulated in the midgut of *A. keiskei*-fed larvae compared with expression in the midgut of *O. javanica*- and *F. vulgare*-fed larvae (Fig. 6B).

Discussion

In this study, we examined the biological activity of the metabolites in the frass extracts from *P. machaon* larvae fed *A. keiskei*, *O. javanica* or *F. vulgare* using a human cancer cell line (HCT116) and we analysed the metabolic processing of these metabolites in the larvae. *P. machaon* metabolites included in the chloroform extract from frass of *A. keiskei*-fed larvae inhibited cell viability and proliferation only in HCT116 cells. Notably, the fractions separated from the chloroform extract of *A. keiskei* leaves did not affect HCT116 viability (Supplementary Fig. 2). Therefore, our results suggest that the metabolites from the frass might reflect a change in chemical structure and biological activity of *A. keiskei* metabolites when processed by the *P. machaon* larval metabolic system. *A. keiskei* contains several bioactive chalcones that are prenylated or geranylated at the 5'-position [15]. Xanthoangelol (5'-geranylated chalcone) induces apoptosis in the human leukaemia cell line K562 more strongly than isobavachalcone (5'-prenylated chalcone), whereas these compounds have no effect on human umbilical vein endothelial cells (HUV-EC) [42]. Xanthoangelol also suppresses the cell viability of the human leukaemia cells HL60, melanoma cells (CRL1579, AZ521) and human stomach cancer cells (KATO III); however, it does not inhibit A549 cell viability [43, 44]. Taken together, these findings indicate that the anticancer activity of 5'-prenylated chalcones changes depending on their modification. Therefore, we speculated that a *P. machaon* larval metabolite produced from an *A. keiskei* chalcone may have inhibited cell viability and proliferation, but only in the HCT116 cell line. The metabolism of chalcones in herbivorous insects awaits further investigation.

It has been reported that the 3'-prenylated chalcone xanthohumol is metabolised by CYP, and that its metabolites are excreted as faeces in rats [45, 46]. In this study, PCA indicated that CYP genes expressed in the *A. keiskei*-fed larval midgut exhibited distinctive expression patterns from those in the *O. javanica*- or *F. vulgare*-fed larval midguts. Additionally, we found that the expression of CYP6B2 genes was significantly upregulated in the midgut of *A. keiskei*-fed larvae compared with expression in the midgut of *O. javanica* and *F. vulgare*-fed larvae, and chalcones, including phenolics and flavonoids, were found only in *A. keiskei*. The CYP enzymes of the CYP6B subfamily from Papilionidae, including CYP6B1 and CYP6B3 from *P. polyxenes*, CYP6B17 and CYP6B21 from *P. glaucus*, and CYP6B33 from *P. multicaudata*, metabolise furanocoumarins [13, 47–49]. Of these, CYP6B1 metabolises more than 20 times more xanthotoxin (furanocoumarin) than that of CYP6B3 [48]. CYP6B1 and CYP6B3 mRNA are highly

expressed in the larval midgut when xanthotoxin is administered to *P. polyxenes* larvae [50]. Although some Papilionidae that use furanocoumarin-containing plants have the CYP6B2 gene, the metabolic products of CYP6B2 have not been identified [51]. Our results suggest that CYP6B2 in *P. machaon* larvae might metabolise furanocoumarin and chalcones from the *A. keiskei* host plant. Therefore, chalcones from *A. keiskei* might be metabolised by CYP in *P. machaon* larvae as well as in mammals.

Conclusion

The frass of *P. machaon* larvae reared on *A. keiskei* contain a metabolite that prevents cell division and induces apoptosis in human colon HCT116 cells. When we investigated the mechanism by which this metabolite was produced, the CYP6B2 gene appeared to be related to the metabolism of *A. keiskei* tissues containing chalcones. The metabolites from the plant undergo conversion of their biological activity through the *P. machaon* larval metabolic system (Fig. 7). These results indicate that the metabolites from *P. machaon* may be useful as pharmaceutical agents against human colon cancer. Additionally, it might be possible to use insect metabolic enzymes for the chemical structural conversion of plant-derived compounds with complex structures. Taken together, our results contribute to the future construction of a novel biotechnology system that entails creating potential pharmaceutical candidates using metabolic enzymes from *P. machaon* larvae.

Methods

Insects and sample collection

P. machaon larvae and eggs were collected at the Fuchu campus of the University of Agriculture and Technology. *P. machaon* larvae were reared on the leaves of *A. keiskei, O. javanica* or *F. vulgare* plants cultivated at the Fuchu campus. Larvae were maintained at 25°C under a 16-h light/8-h dark cycle. The larval frass and host plant leaves were collected and stored at -30°C until use.

Extraction of metabolites from larval frass and leaves

We placed each sample on a glass petri dish and freeze-dried it with a lyophiliser (VD-250F, TAITECH Co., Ltd., Saitama, Japan) for 24 h. We pulverised each sample and weighed it using a Mettler balance. We transferred the weighed sample to a 1000 mL Erlenmeyer flask and added 3 volumes of n-hexane (Hex; Wako Pure Chemical Industries, Ltd., Osaka, Japan). We allowed the mixture to sit for 24 h at room temperature (RT) and then filtered it. We transferred the pellet to a 1000 mL Erlenmeyer flask, added 3 volumes of Hex, left it for 24 h at RT, and then filtered it. We repeated this operation one more time and transferred the filtrate to an eggplant flask for evaporation. We transferred the pellet to a 1000 mL Erlenmeyer flask and added 3 volumes of chloroform (CHCl3; Wako Pure Chemical Industries, Ltd.) to the pellet. Then, we performed the same operation with Hex. We then transferred the pellet to a 1000 mL Erlenmeyer flask and added 3 volumes of methanol (MeOH; Wako Pure Chemical Industries, Ltd.) to the pellet. Then, we performed the same operation with Hex. We concentrated these filtrates, including n-hexane, CHCl3 or MeOH, by a rotary evaporator (N-1110 N, Tokyo Scientific Instruments Co., Ltd., Tokyo,

Japan). Finally, we collected each extract in a screw tube (No. 7, Marem Co., Ltd., Osaka, Japan), kept the extracts in a vacuum desiccator for 3–5 days to completely remove each organic solvent and then weighed the obtained extract. We shielded the screw tubes containing the extracts from light with aluminium foil and stored them at 4°C until use. We referred to the extract with n-hexane as Hex. ext., the extract with CHCl3 as Chl. ext., and the extract with MeOH as Met. ext. Supplementary Table 2 shows the weight of samples before and after freeze-drying and the weight of their extracts.

Thin-layer chromatography (TLC)

To separate and compare the metabolites from the larval frass or the host plants, each extract dissolved with extraction solvent at 10 mg/mL was applied to a silica gel TLC aluminium plate (Merck®, Darmstadt, Germany) in 1 µL (chloroform extract) or 2 µL (hexane and methanol extract) using a glass capillary. A mixed solvent was used as the eluent: chloroform-methanol (15:1, v/v) for the hexane extract, chloroform-methanol (10:1, v/v) for the chloroform extract and acetic acid-butanol-H_2O (1:8:1, v/v) for the methanol extract. For detection of spots, we first used UV light to mark the spots (256 nm; left, 366 nm; right). Next, the plates were stained with sodium phosphomolybdate n-hydrate in ethanol (9%, v/v) to detect spots without UV exposure. We used the position of each spot as a relative to the front (Rf) value. The Rf value was calculated as follows: distance from baseline travelled by the solute divided by distance from baseline travelled by the solvent (solvent front).

Separation of metabolites using open column chromatography

Chloroform extracts were separated on a silica gel (Kanto Chem., Tokyo, Japan) column (column size 30 x 300 mm and 170 mm height silica gel) with an eluting solvent of chloroform-methanol (10:1, v/v). These fractions dissolved in 300 mL solvent were collected in 26 mL glass test tubes to yield 11 fractions from the frass of *A. keiskei*-fed larvae, 3 fractions from the frass of *O. javanica*-fed larvae, 4 fractions from the frass of *F. vulgare*-fed larvae and 6 fractions from *A. keiskei* leaves based on the TLC profile. Fr. 1, separated from the chloroform extract from the frass of *A. keiskei*-fed larvae, inhibited HCT116 viability. Thus, Fr. 1 was further separated using a silica gel (FUJI SILYSIA Chemical LTD., Aichi, Japan) column (column size 15 x 300 mm, 170 mm height silica gel) and by eluting solvent of chloroform-methanol (18:1, v/v). The fractions dissolved in 60 mL of solvent were collected to yield 2 fractions based on the TLC profile.

Cell culture

HCT116 (RCB2979), A549 (RCB0098), HeLa (RCB0007), HepG2 (RCB1886), MIA PaCa2 (RCB2094), TGBC1TKB (RCB1129) and HFSKF-II (RCB0698) cells were obtained from the Riken Cell Bank (Riken Tsukuba, Japan). A549, HCT116, HeLa, HepG2 and MIA PaCa2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Nacalai Tesque, Kyoto, Japan) supplemented with 10% foetal bovine serum (FBS, Gibco, USA). TGBC1TKB cells were maintained in DMEM supplemented with 5% FBS. HFSKF-II was maintained in Ham's F-12 (Nacalai Tesque, Kyoto, Japan) supplemented with 15% FBS. One

hundred units/mL penicillin–streptomycin (Fujifilm Wako Pure Chemical Corp., Osaka, Japan) was added to DMEM and Ham's F-12. Cell lines were cultured at 37°C in a humidified chamber containing 5% CO_2.

Cell viability assays

For the cell viability assays, cells were seeded in 96-well plates (HepG2: 700 cells per well; A549, HCT116, HeLa and TBC1TKB: 1000 cells per well; MIA PaCa2 and HFSKF-II: 2000 cells per well; experiment performed in triplicate). Then, after 24 hours of incubation, medium containing each fraction dissolved in dimethyl sulfoxide (DMSO; Wako Pure Chemical Industries, Ltd.) was added to each well at final concentrations of 0.05, 0.5 and 5 μg/mL, whereas medium containing only DMSO was added to each control well at a final concentration of 0.5% (v/v). Forty-eight hours later, 10 μL/well WST-1 reagent (Takara Bio., Shiga, Japan) or WST-8 reagent (Doujin Chemical, Kumamoto, Japan) was added. After incubation for 4 hours, the absorbance at 450 nm and 620 nm was measured using a microplate reader, iMark™ (Bio-Rad Laboratories Inc., Hercules, California, U.S.A.) or Gene5 (BioTek Instruments, Winooski, Vermont, U.S.A). The value of the absorbance at 620 nm, which was the control wavelength, was subtracted from the value of the absorbance at 450 nm. As a medium blank, 10 μL of WST-1 or WST-8 reagent was added to a well containing only medium without cells, and the average of the absorbance of this well was subtracted from all other values obtained. Finally, the cell viability was calculated using the following formula.

Cell viability (%) = $([Abs_{sample}-Abs_{blank}]/[Abs_{control}-Abs_{blank}]) \times 100$

Cell proliferation assay

For the BrdU cell proliferation assay, 1000 HCT116 cells per well were cultured in a 96-well plate for 24 hours. Then, the medium including Fr. 1 dissolved in DMSO was diluted with medium, and the medium was added to a well to a final concentration of 0.2, 1 and 5 μg/mL. After incubation for 24 hours, 20 μL of BrdU reagent-containing medium was added to the measuring well, whereas 20 μL of BrdU reagent-free medium was added to a well as background. Finally, after culturing for 24 hours, cell proliferation was evaluated using a BrdU assay kit (Abcam, Cambridge, U.K). according to the manufacturer's protocols. The absorbance of background wells was subtracted from all other values obtained.

Cell morphological observation

To assess the morphological changes in HCT116 cells after treatment with Fr. 12 and Fr. 13, the cells were cultured in a 24-well plate for 24 hours. Then, Fr. 12, Fr. 13 (5 μg/mL), 0.5% (v/v) DMSO as a negative control or 5 μM cisplatin (CDDP; Fujifilm Wako Pure Chemical Corp., Osaka, Japan) as a positive control. Forty-eight hours later, the cell morphology was observed under a microscope, EVOS® FLoid® Cell Imaging Station (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.)

Comparisons of host plant components

To compare the chemicals contained in each host plant, we utilised TUAT-insecta [19], which is a database integrating information on herbivorous insects and their host plants with information on

biological activity and chemicals. Information on *A. keiskei*, *O. javanica* and *F. vulgare* was obtained using their scientific names to search the TUAT-insecta database.

RNA sequencing for HCT116 cells treated with Fr. 12

We purified total RNA from HCT116 cells treated with Fr. 12 (5 μg/mL) or 0.1% (v/v) DMSO using TRIzol reagent (Thermo Fisher Scientific, USA) and a PureLink® RNA Extraction Kit (Thermo Fisher Scientific, Japan) according to the manufacturer's protocol. Then, we used an Agilent TapeStation 2200 (Agilent Technologies, Santa Clara, CA) to assess the RNA quality. cDNA library construction of total RNA from these samples (100 ng) was carried out using the TruSeq® Stranded mRNA Library Preparation Kit (Illumina, Inc., San Diego, CA) according to the manufacturer's instructions. Finally, the libraries (100 bp, paired-end) were sequenced using the Illumina NovaSeq6000 platform.

Gene enrichment analysis in HCT116 cells treated with Fr. 12

To analyse the differentially expressed genes (DEGs) in HCT116 cells treated with Fr. 12, FASTQ files were assessed by TrimGalore! version 0.6.6 (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Then, these transcript data were mapped to the human transcript reference (GRCh38.p14) obtained from NCBI (accessed on 5th September 2022) using Salmon version 1.9.0 (http://salmon-tddft.jp/download.html) to obtain transcripts per kilobase million (TPM) and count data. Next, DEGs in Fr. Twelve-treated HCT116 cells compared to the control were determined with the count data by using R version 4.2.1 (https://www.r-project.org/) and the limma voom package (version 3.16) in TCC-GUI version 1.0 [51]. Finally, we performed functional analysis using the gene list of DEGs with the Metascape online tool (metascape.org) [52]. Gene enrichment analysis was carried out with Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway, GO Biological Processes, Reactome Gene Sets, Canonical Pathways, CORUM and WikiPathways. Terms with a p value < 0.01, a minimum count of 3, and an enrichment factor > 1.5 (the enrichment factor is the ratio between the observed counts and the counts expected by chance) were collected and grouped into clusters based on their membership similarities. The most statistically significant term within a cluster were chosen by Metascape to represent the cluster. Three samples in each group were used for RNA sequencing analysis.

RNA-Seq analysis in **P. machaon** larval midgut and fat body

Total RNA from the midgut and fat body of *P. machaon* 5th larvae reared on *A. keiskei*, *O. javanica* or *F. vulgare* was purified, and RNA-seq analysis was performed as mentioned above. All expressed genes were calculated using *P. machaon* reference transcripts downloaded from NCBI (ilPapMach1.1, accessed on 5th September 2022). To investigate the *CYP* genes, we used the reference transcript data as follows: (1) translated the reference transcript into amino acid sequences using TransDecoder (https://github.com/TransDecoder/TransDecoder/releases) version 5.5.0; (2) searched for the domain of CYPs (ID: PF00067) in the Protein Family (Pfam) database by performing HMMER (hmmsearch)

(http://hmmer.org) and converted them for the Basic Local Alignment Search Tool (BLAST) database (makeblastdb) version 2.13.0; (3) extracted the sequence possessing the *CYP* domain from the BLAST database; (4) identified *CYP*s with amino acid sequences and their functions for insect proteins obtained from UniProt using a systematic BLAST search (blastx). Finally, we integrated the TPM from the Salmon results and the annotation of *CYP*s from the BLAST results based on RefSeq IDs. PCA was carried out using R software version 4.2.1 with TCC-GUI version 1.0.

Data availability

The RNA sequencing datasets generated and/or analysed during the current study are available in the Sequence Read Archive, DNA Data Bank of Japan repository, under the following accession IDs: HCT116 treated with Fr. 12 (SRA accession numbers: DRR428503, DRR428504, and DRR428505), HCT116 treated with DMSO (SRA accession numbers: DRR428506, DRR428507, and DRR428508), *P. machaon* larval midgut and fat body from larvae eating *A. keiskei*, (DRR428498, and DRR428497), *P. machaon* larval midgut and fat body from larvae eating *O. javanica* (DRR428502, and DRR428501), and *P. machaon* larval midgut and fat body from larvae eating *F. vulgare* (DRR428500, and DRR428499).

Abbreviations

CYP
Cytochrome P450
TLC
Thin-layer chromatography
DEG
Differentially Expressed Gene
GO
Gene Ontology
KEGG
Kyoto Encyclopedia of Genes and Genomes
PCA
Principal component analysis
TPM
Transcripts per million

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The RNA sequencing datasets generated and/or analysed during the current study are available in the Sequence Read Archive, DNA Data Bank of Japan repository, under the following accession IDs: HCT116 treated with Fr. 12 (SRA accession numbers: DRR428503, DRR428504, and DRR428505), HCT116 treated with DMSO (SRA accession numbers: DRR428506, DRR428507, and DRR428508), *P. machaon* larval midgut and fat body from larvae eating *A. keiskei*, (DRR428498, and DRR428497), *P. machaon* larval midgut and fat body from larvae eating *O. javanica* (DRR428502, and DRR428501), and *P. machaon* larval midgut and fat body from larvae eating *F. vulgare* (DRR428500, and DRR428499).

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

M.N., R.J.S., and H.T. conceived and designed the study. M.N., H.B., and T.S. performed the experiments and collected data. Y.K. helped analyse the ingredients. Writing—original draft: M.N. and H.T. All authors discussed the data and helped with manuscript preparation. H.T. supervised the project. All authors read and approved the final manuscript.

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Tables

Tables 1 and 2 are available in the Supplementary Files section.

Figures

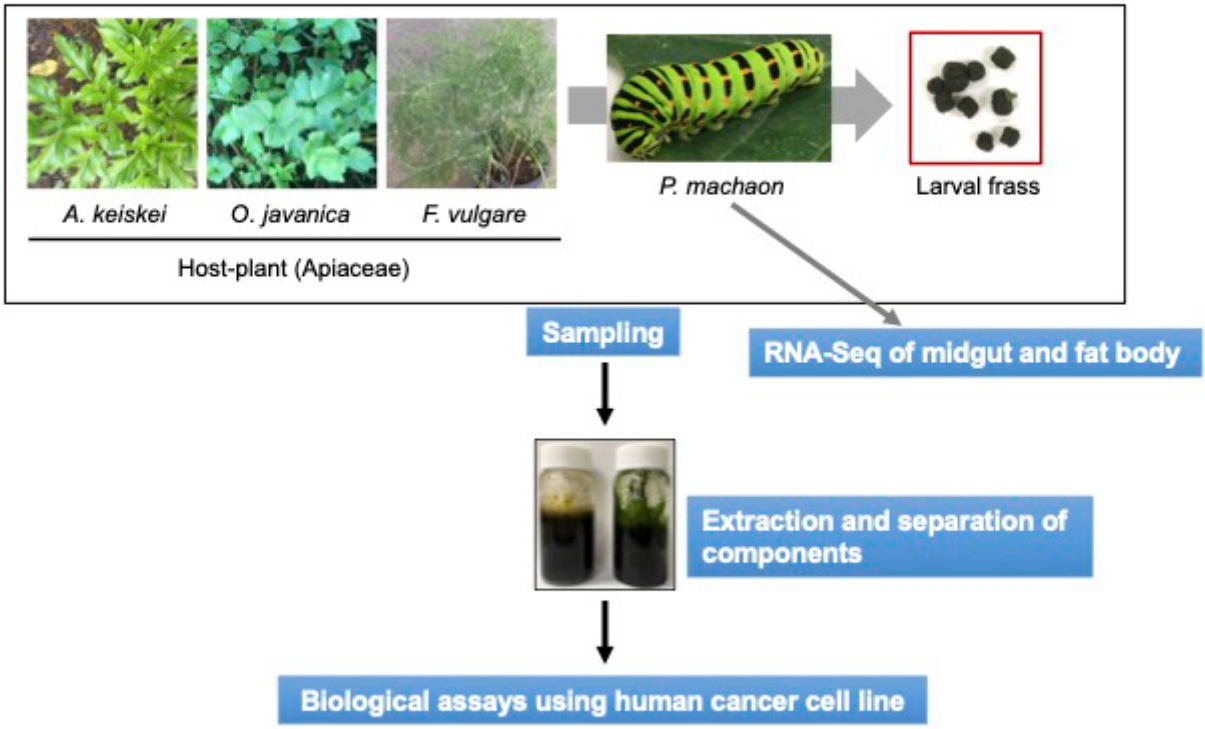

Figure 1

Outline of this study. *P. machaon*larvae ate three kinds of host plants, and then the metabolites were extracted from the frass of each host plant group using three kinds of organic solvents, and the fractions were separated using open column chromatography. The fractions were examined for biological activity using human cancer cell lines.

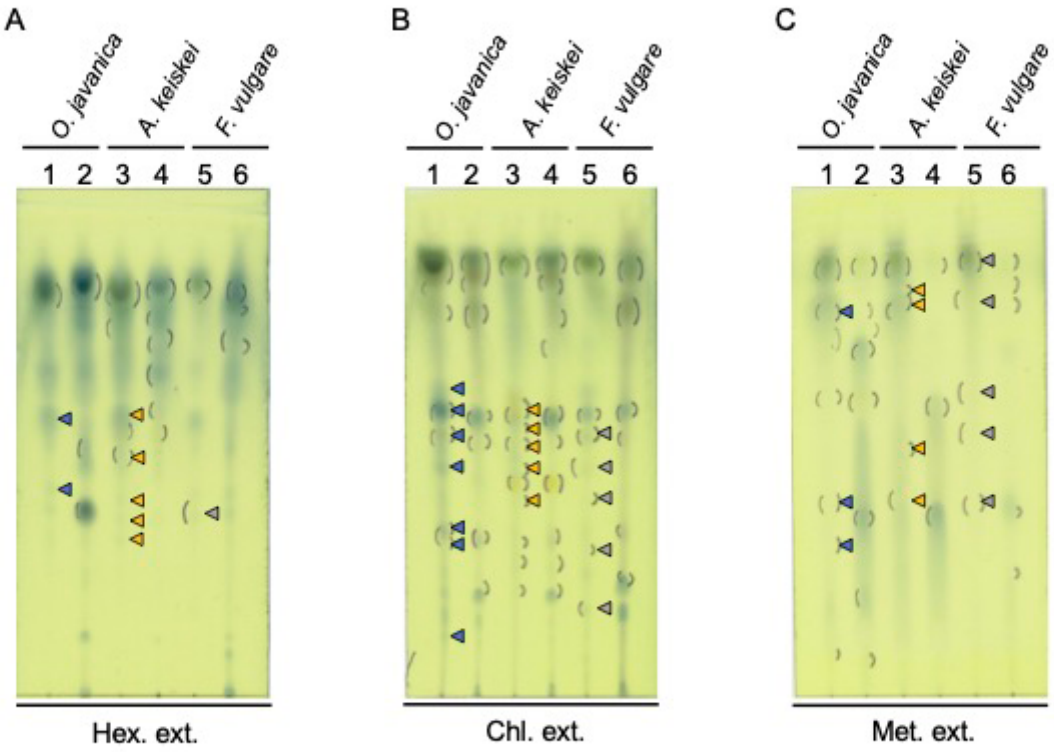

Figure 2

Comparison of the metabolites included in each extract using TLC analysis. The different spots between extracts from frass and extracts from hostplants areshown as arrowheads for Hex. ext. (A), Chl. ext. (B) and Met. ext. (C).

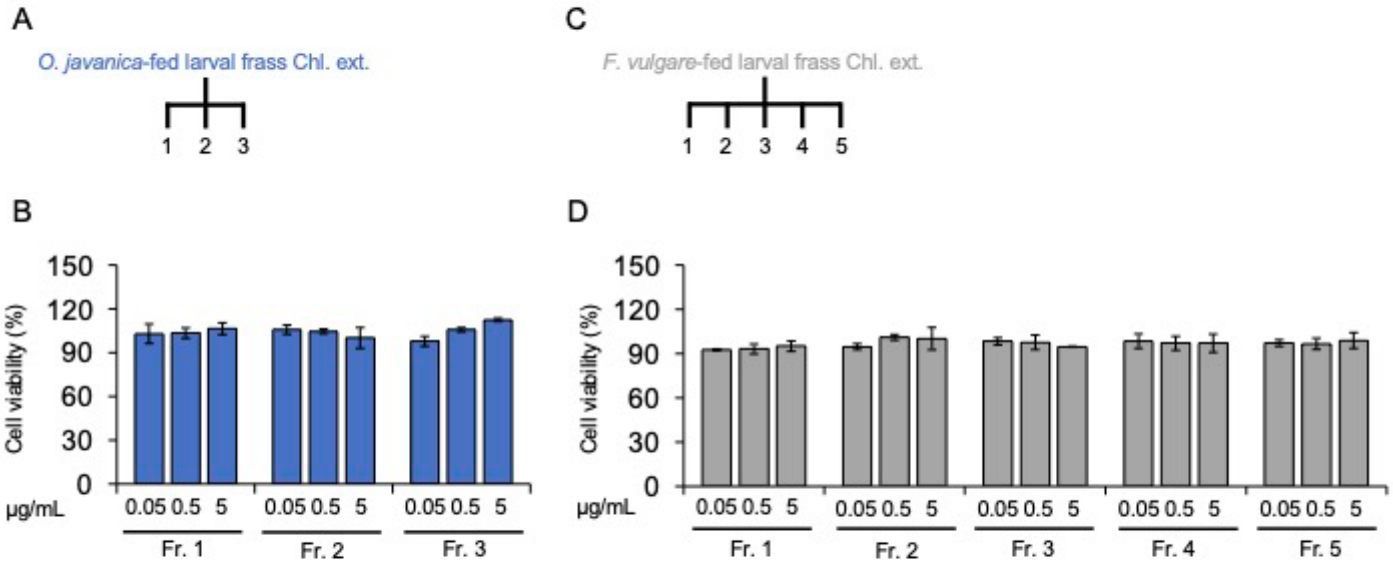

Figure 3

Cell viability of HCT116 cells treated with fractions. (A, B) Fractions separated from Chl. ext. of *O. javanica*-fed larval frass (A) and their effect on HCT116 viability (B). (C, D) Fractions separated from Chl. ext. of *F. vulgare*-fed larval frass (C) and their effect on HCT116 viability (D). Cellviability was measured using the WST-8 assay. Error bars represent the mean ± SDfrom three biological replicates.

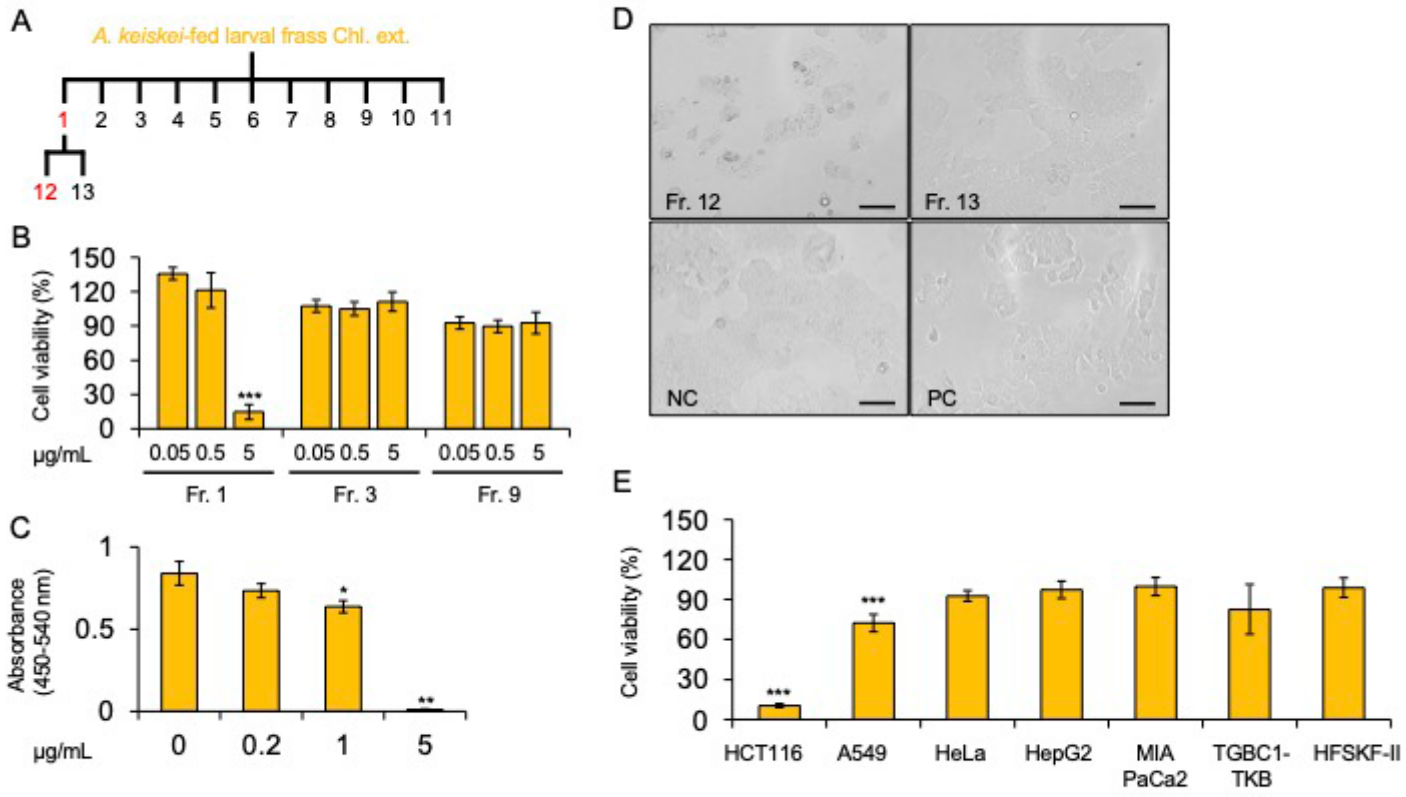

Figure 4

Biological activity on HCT116 by fractions separated from *A. keiskei*-fed larval frass. (A) Thirteen fractions separated from Chl. ext. using open column chromatography. (B) Cell viability of HCT116 cells treated with Fr. 1, Fr. 3 and Fr. 9. (C) BrdU assay in HCT116 cells after treatment with Fr. 1. The absorbance indicates inhibition of cell proliferation. (D) Morphological observation of HCT116 cells treated with 5 μg/mL of Fr. 12 or Fr. 13. DMSO (0.5%, v/v) was used as a negative control (NC), and 5 μM cisplatin (CDDP) was used as a positive control (PC). (E) Cell viability of A549 (lung cancer), HeLa (cervical cancer), HepG2 (hepatic cancer), MIA PaCa2 (pancreatic cancer), TGBC1TKB (gallbladder cancer) and HFSKF-II (human fibroblasts derived from foetal skin) cells treated with 5 μg/mL of Fr. 12. Cell viability was determined by WST-1 assay. Error bars represent the mean ± SD from three biological replicates. Significant differences from the control were analysed with Student's t test, *P < 0.05, **P < 0.01, ***P < 0.001. Scale bars = 100 μm.

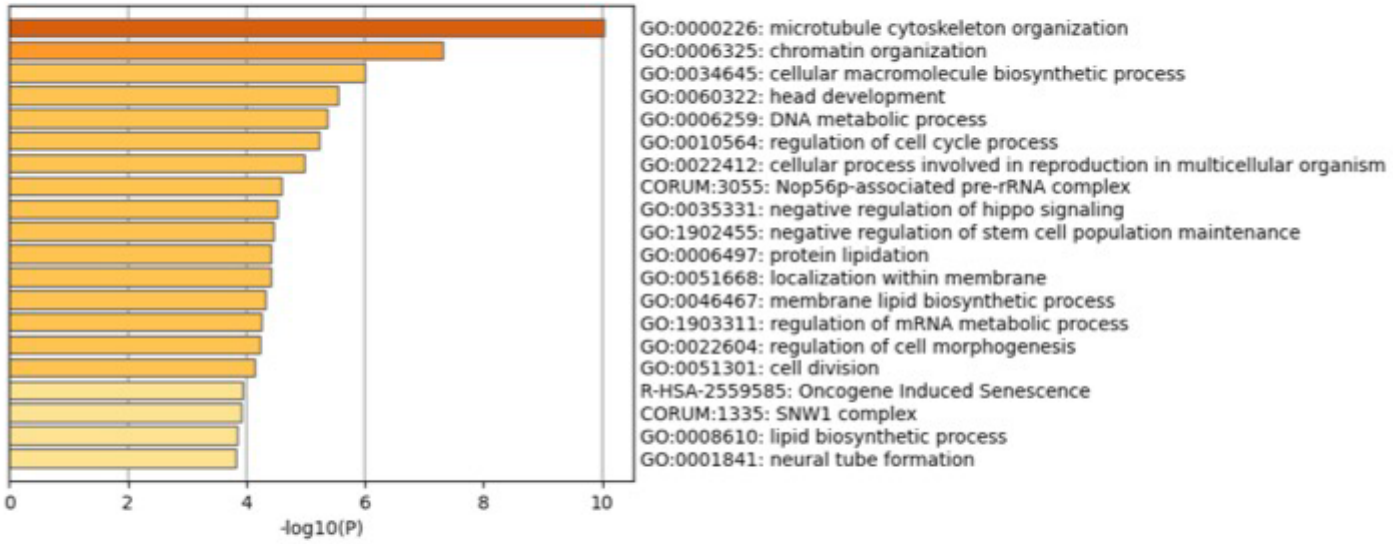

Figure 5

Gene enrichment analysis of DEGs in HCT116cellstreated with Fr. 12. Gene enrichment analysis of differentially expressed transcripts in HCT116 cellstreated with Fr. 12 using Metascape. A bar graph for enriched terms across the input transcript lists; different coloured bars indicate different P values.

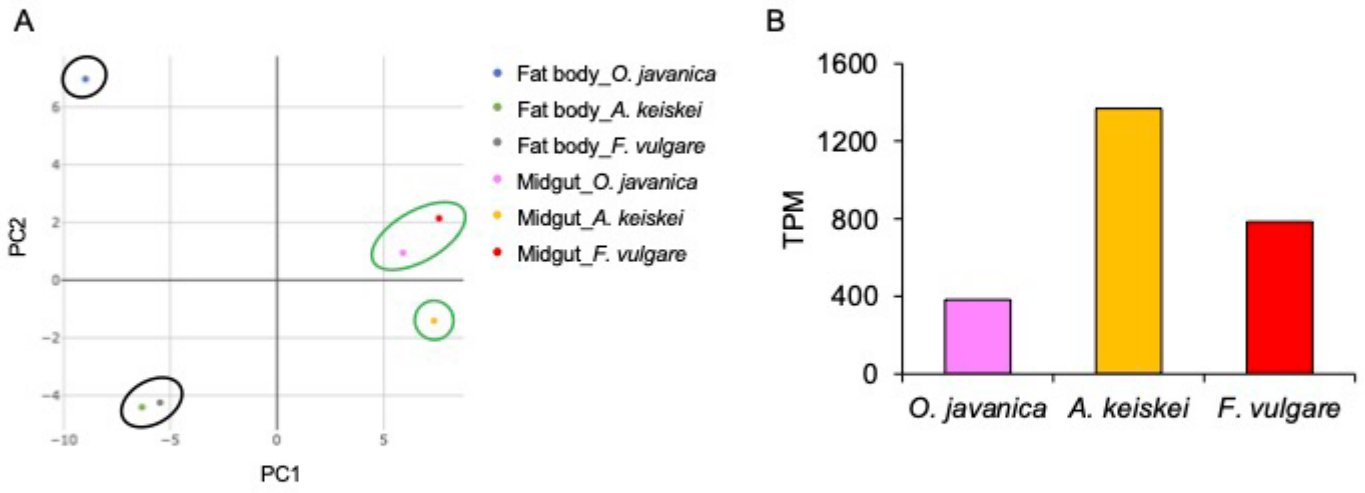

Figure 6

Expression of *CYPs* in the *P. machaon* larval midgut and fat body.(A) PCA for the CYP genes expressed in the larval midgut and fat body fed on *A. keiskei*, *O. javanica* or *F. vulgare*. Four groups were generated; theblack circle indicates the fat body, and the green circle indicates the midgut. (B) The graph shows the expression of *CYP6B2* in the larval midgut with TPM values analysed by Salmon (n = 1).

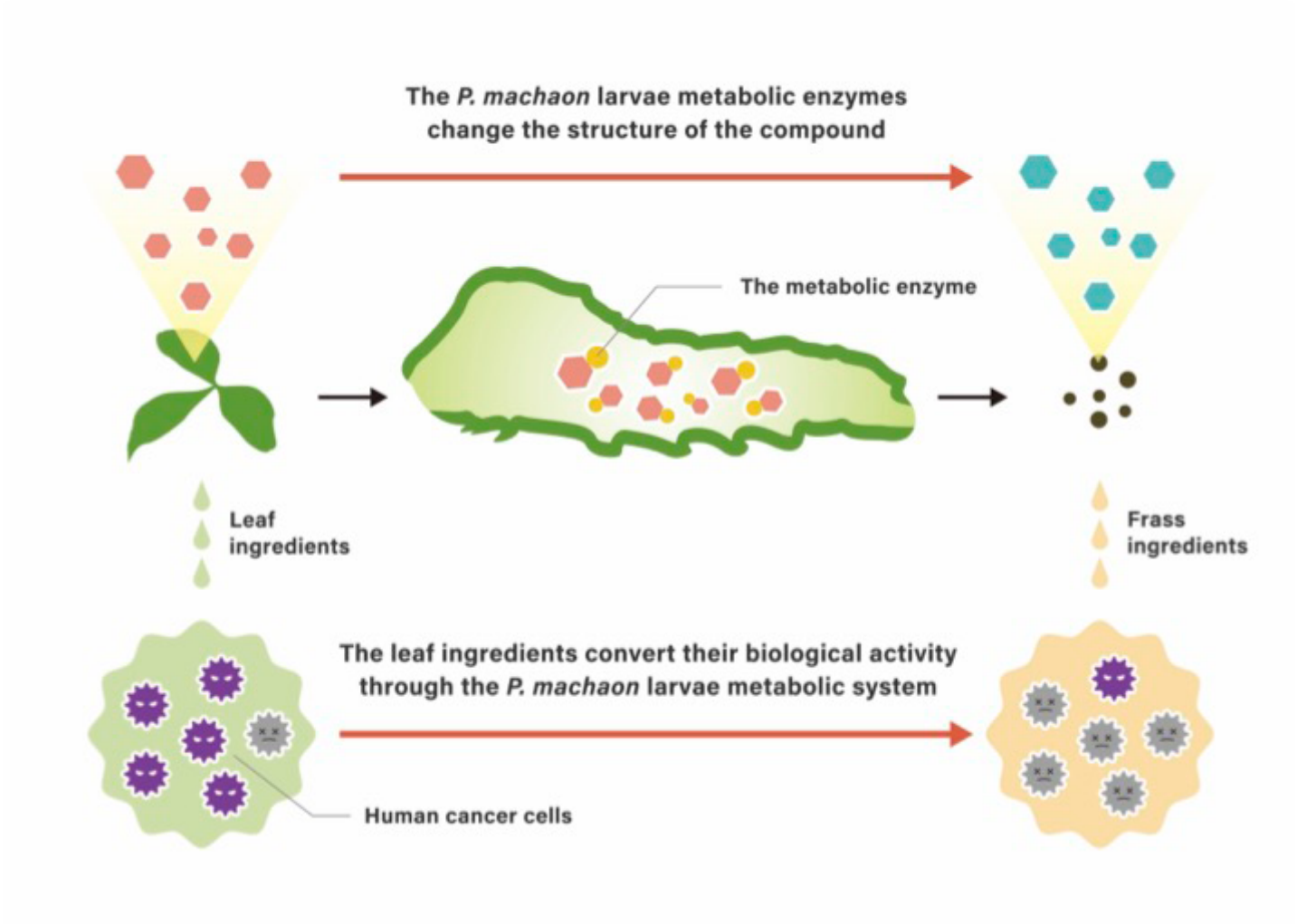

Figure 7

The predicted metabolic process for the metabolites derived from host plants. *P. machaon* larvae eat host plants, andtheir metabolic enzymes, CYPs, change the structure of compounds included in the host plant tissues. The metabolites from the host plants undergo conversion of their biological activity through the *P. machaon* larval metabolic process.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryMaterialsforBMCGenomics.pdf
- Table1.xlsx
- Table2.xlsx
- SupplementaryfigureandtableLegend.docx