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Invertebrates living in polluted environments are potential source of novel anticancer agents

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ABSTRACT: One of the leading causes of mortality and morbidity worldwide, cancer is a major medical concern with 14.1 and 8.2 million cases of new cancer cases and death cases recorded in 2012 alone. The number of deaths related to cancer are still on the rise despite various treatment options. Hence, there is a need for the identification of anticancer agents for treatment. This study focused on identifying anticancer agents from invertebrates thriving in polluted environments; *Acheta domesticus* (cricket), *Anadara granosa* (blood clam), *Blaptica dubia* (cockroach), *Penaeus monodon* (tiger prawn) and *Scolpendra subspinipes* (centipede) respectively. We hypothesized that gut microbes of animals/pests living in polluted environments such as cockroaches are a potential source of novel anticancer agents. To evaluate this hypothesis, invertebrates were dissected and their gut bacteria were identified and conditioned media were prepared. The conditioned media were used to conduct cytotoxicity assays, cell survival assays and cell growth assays, against two cancer cell lines (cervical and prostate cancer cells) as well as normal cells (HaCaT, aneuploid immortal keratinocyte). The results revealed that conditioned media from tiger prawn (*Pseudomonas oryzihabitans*) and centipede (*Kocuria varians*) exhibited significant cytotoxic and growth inhibitory effect against the cell lines tested. However, further studies need to be conducted to identify and characterize the active molecule(s).

KEYWORDS: Insects; pests; cancer; treatment; cytotoxicity; growth inhibition.

1. INTRODUCTION

Despite therapeutic advances and supportive care, cancer remains the leading cause of morbidity and mortality worldwide. According to the International Agency for Research on Cancer (IARC), there were 14.1 million new cancer cases, 32.6 million pre-existing cancer patients and 8.2 million deaths worldwide in 2012 alone [1,2]. By 2030, the global cancer burden is expected to nearly double, growing to 21.4 million cases and 13.2 million deaths. These numbers have remained significant in spite of available treatments for cancer including chemotherapy, radiation therapy, stem cell therapy, immunotherapy, targeted therapy, hormone therapy and surgery, highlighting the need to identify novel anticancer agent(s) [3-5].

Cancer is often linked to environmental pollutants, chemicals, infectious agents, genetics, hormones and radiation. With this in mind, it is important to note that pests, such as cockroaches can tolerate high levels of radiation, and thrive in unhygienic conditions with exposure to heavy metals. Other animals, such as crocodiles thrive from feeding on germ-infested rodents, exposed to heavy metals such as arsenic, cadmium, cobalt, chromium, mercury, nickel, lead, selenium, endure high levels of radiation, are among the very few species to survive the catastrophic Cretaceous-Tertiary extinction event, and yet live up to a 100 years [6-11]. Thus, it is reasonable to hypothesize that species such as crocodiles and cockroaches have developed mechanisms to defend themselves from noxious agents. We recently tested this hypothesis using an adult crocodile and showed that the organ lysates and sera of crocodile exhibited potent anticancer properties [12,13]. There are two logical reasons to explain these findings: (i) animals/pests living in polluted environments have evolved a strong immune system to counter cancer development, and/or, (ii) gut bacteria of animals/pests living in polluted environments produce anticancer molecule(s). In the present study, we selected several invertebrate species including *Acheta domesticus, Anadara granosa, Blaptica dubia, Penaeus*

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monodon and *Scolpendra subspinipes,* (Table 1) and tested their body lysates (Fig. 1) as well as their gut bacteria for potential anticancer properties.

Table 1. The species, scientific classification, habitat and diet of animals used in this study.

Animal (species)	Scientific classification	Habitat	Diet
Scolpendra subspinipes (centipede)	Kingdom: Animalia Phylum: Arthropoda Class: Chilopoda Order: Scolopendromorpha Family: Scolopendridae Genus: *Scolopendra* Species: *S. subspinipes*	Moist condition to survive on, mostly live in the soil.	Eat anything that is soft-bodied and fits in their mouth (E.g: Spiders, Lizards, Rodents, etc.)
Penaeus monodon (tiger prawn)	Kingdom: Animalia Phylum: Arthropoda Subphylum: Crustacea Class: Malacostraca Order: Decapoda Suborder: Dendrobranchiata Family: Penaeidae Genus: *Penaeus* Species: *P. monodon*	Shore areas and mangrove estuaries.	Crabs, shrimps, mollusks, algae and plant material and dead/decaying organic matter.
Acheta domesticus (cricket)	Kingdom: Animalia Phylum: Euarthropoda Class: Insecta Order: Orthoptera Suborder: Ensifera Family: Gryllidae Genus: *Acheta* Species: *A. domesticus*	Woodlands, caves, pastures, fields, below logs and rocks.	Rotting plant matter, leaves, fungi, fruit, insects and bugs.
Anadara granosa (blood clam)	Kingdom: Animalia Phylum: Mollusca Class: Bivalvia Subclass: Pteriomorphia Order: Arcoida Family: Arcidae Genus: *Tegillarca* Species: *T. granosa* or *A. granosa*	20 meters deep waters, beaches, sea shore.	Detritus, unicellular algae and phytoplankton.
Blaptica dubia (dubia roach)	Kingdom: Animalia Phylum: Arthropoda Class: Insecta Order: Blattodea Family: Blaberidae Genus: *Blaptica* Species: *B. dubia*	Sewage, manholes, gardens, animal manure piles.	Fruits and grains but feeds on any left overs, paper, rubbish.

2. RESULTS

2.1. A spectrum of bacteria was identified from cricket, blood clam, dubia roach, prawn and centipede

A spectrum of bacteria was isolated from the gut of the various dissected invertebrates (Table 2).

The bacterial species isolated from cricket were *Klebsiella pneumoniae, Pantoea* sp., *Staphylococcus lentus* and *Staphylococcus xylosus.* While, *Micrococus* spp., *Pseudomonas oryzihabitans* and *Staphylococcus sciuri* were isolated from the gut of blood clam. The bacteria isolated from dubia roach include *Staphylococcus hominis* and *Staphylococcus xylosus. Acinetobacter baumannii, Pseudomonas luteola, Pseudomonas oryzihabitans* and *Staphylococcus xylosus* were isolated from tiger prawn. *K. varians, Micrococcus* spp., and *Staphylococcus lentus* were isolated from centipede.

2.2. Gut bacteria from selected invertebrates exhibited anticancer properties

From above, bacterial conditioned media were tested for anticancer properties using normal and cancer cell lines. The results revealed that gut bacteria isolated from cockroaches showed anticancer effects (Table 3). For example, conditioned media of *S. xylosus* from cockroach gut showed reduced growth against PC3 cells compared with HaCat cells ($P<0.05$) (Table 3). However, conditioned media of *S. xylosus* showed no effects against Hela cells ($P>0.05$) (Table 3).

Figure 1. The dissection procedures conducted for the vertebrates used. A: *Anadara granosa*, B: *Acheta domesticus*, C: *Blaptica dubia*, D: *Penaeus monodon* and E: *Scolpendra subspinipes*.

Table 2. Bacteria isolated from selected invertebrates and their conditioned media.

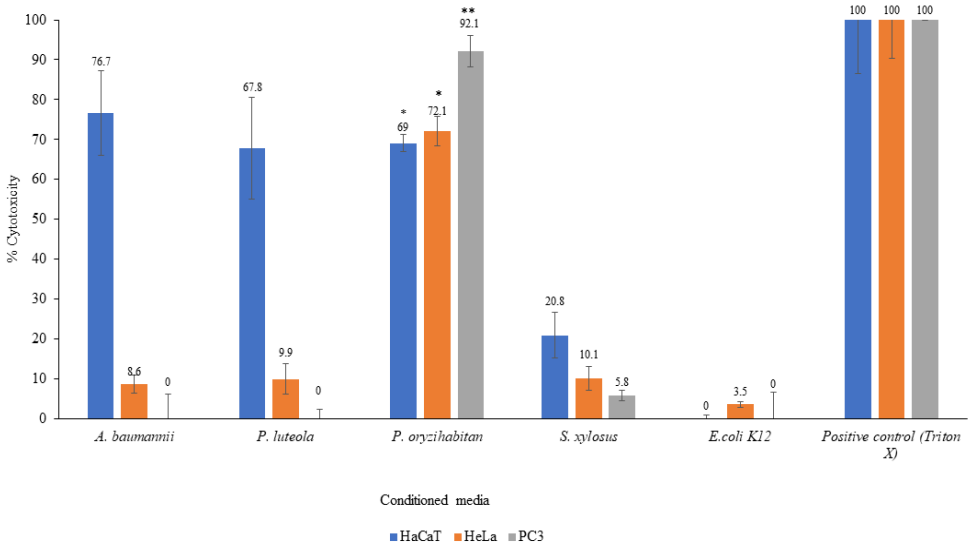
Species	Conditioned media	Bacterial Identity
Scolpendra subspinipes (centipede)	CM1	*Kocuria varians*
	CM2	*Micrococcus spp*
	CM3	*Staphylococcus lentus*
Penaeus monodon (tiger prawn)	CM1	*Acinetobacter baumannii*
	CM2	*Pseudomonas luteola*
	CM3	*Pseudomonas oryzihabitans*
	CM4	*Staphylococcus xylosus*
Acheta domesticus (cricket)	CM1	*Klebsiella pneumoniae*
	CM2	*Pantoea spp1*
	CM3	*Staphylococcus auricularis*
	CM4	*Staphylococcus lentus*
	CM5	*Staphylococcus xylosus*
Anadara granosa (blood clam)	CM1	*Micrococcus spp*
	CM2	*Pseudomonas oryzihabitans*
	CM3	*Staphylococcus sciuri*
Blaptica dubia (dubia roach)	CM1	*Staphylococcus hominis*
	CM2	*Staphylococcus xylosus*
	CM3	*Staphylococcus xylosus*

Table 3. The effects of conditioned media of selected invertebrate species on growth and cell death of various human cancer and normal cells.

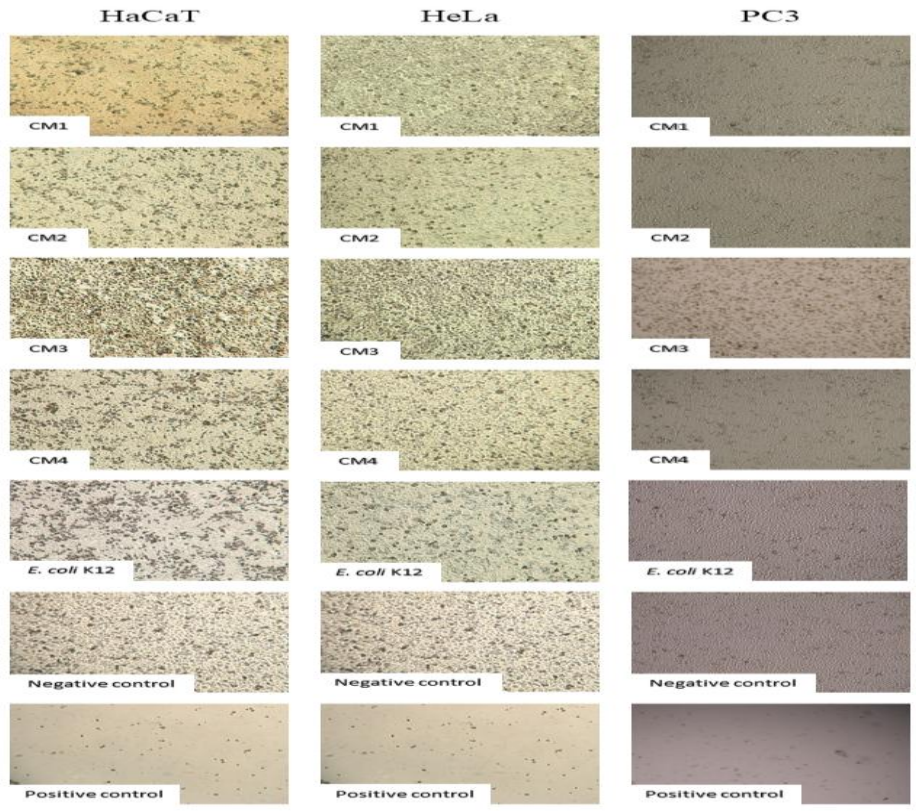
Invertebrates	Bacteria species	% Growth			% Cell Death		
		HeLa	PC3	HaCaT	HeLa	PC3	HaCaT
Control		**100%**			**0%**		
Scolpendra subspinipes (centipede)	*Kocuria varians*	0±0	0±0	0±0	85.2±4.0	100±4.0	100±4.8
	Micrococcus spp.	68.0±12.2	93.9±4.7	70.6±7.4	5.5±1.1	0.6±5.4	2.3±0.7
	Staphylococcus lentus	59.3±5.4	46.5±3.0	43.4±3.4	2.2±1.3	28.7±0.6	16.7±4.8
Penaeus monodon (tiger prawn)	*Acinetobacter baumannii*	56.2±3.3	46.8±7.1	66.1±0.4	8.6±2.3	0±6.0	76.7±10.6
	Pseudomonas luteola	67.9±9.1	47.7±0.6	52.6±0.1	9.9±3.8	0±2.3	67.8±12.7
	Pseudomonas oryzihabitans	0±0.2	0±0.1	0±0.5	72.1±3.7	92.1±4.0	69.0±2.2
	Staphylococcus xylosus	56.9±0.6	80.2±6.0	42.0±7.0	10.1±3.0	5.8±1.3	20.8±5.7
Acheta domesticus (cricket)	*Klebsiella pneumoniae*	60.2±3.5	48.1±0.3	45.8±2.3	1.5±0.8	0±1.1	20±11.7
	Pantoea spp.	72.9±0.7	67.6±2.3	75.8±8.9	3.6±3.4	0±1.6	23.7±7.3
	Staphylococcus auricularis	64.1±1.4	72.0±2.8	48.6±0.4	0.7±1.9	0±0.5	2.3±2.4
	Staphylococcus lentus	55.5±2.7	64.6±4.2	56.7±7.5	1.6±0.1	0±0.7	10.5±6.4
	Staphylococcus xylosus	55.0±2.0	99.1±2.4	53.3±3.5	4.2±6.0	0±0.8	0±7.5
Anadara granosa (blood clam)	*Micrococcus spp.*	49.1±2.3	8.0±3.1	16.3±3.5	1.0±0.2	0±1.8	0.3±4.6
	Pseudomonas oryzihabitans	62.8±2.7	11.9±12.7	0±12.9	0.7±0.8	0±7.3	21.0±3.3
	Staphylococcus sciuri	55.6±7.1	96.5±0.4	48.0±7.8	8.9±3.2	0±5.4	62.8±2.9
Blaptica dubia (dubia roach)	*Staphylococcus hominis*	77.3±2.1	81.6±4.0	70.0±2.5	0.8±0.7	0±1.7	0±0
	Staphylococcus xylosus	65.8±1.8	49.3±6.6	73.1±7.0	2.5±1.1	0±0.8	58.9±0.4
	Staphylococcus xylosus	68.2±8.0	97.5±4.3	77.2±8.5	2±1.4	0±1.2	25.9±2.1

Similarly, conditioned media of *A. baumannii* from prawn showed reduced growth against PC3 cells compared with HaCat cells ($P<0.05$) (Table 3). For cytotoxicity, conditioned media of *P. oryzihabitans* from prawn showed higher cytotoxicity against PC3 cells compared with HaCat cells (Table 3). In contrast, conditioned media of *K. varians* exhibited broad-spectrum cytotoxic effects (Table 3), while other lysates showed no or minimal effects. The representative images for HaCaT, HeLa and PC3 cells further confirm that CM1, CM2 and CM4 but not CM3 from tiger prawn did not yield any significant cytotoxic effects against cell lines tested (Fig. 2A,B,C). The cells treated with CM3 were observed to be smaller and rounder in shape. Furthermore, the cell staining indicated that cells were not affected post-treatment with CM1, CM2 and CM4 from prawn, similar to the negative controls, indicating that cells were viable, except for cells treated with CM3 (Fig. 2C).

A)



B)



C)

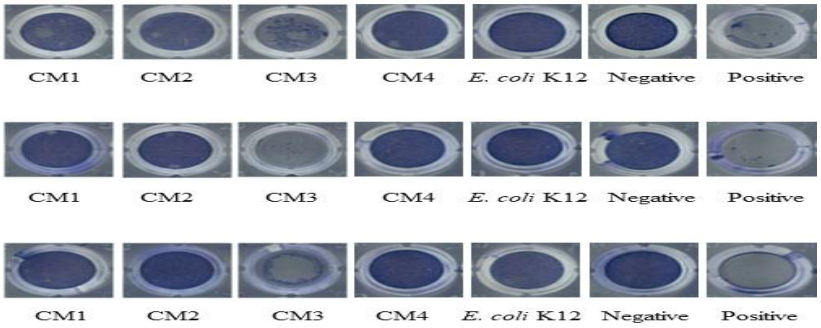


Figure 2A: The cytotoxic effect of the conditioned media from tiger prawn against HaCaT, HeLa and PC3 cells. Confluent HaCaT, HeLa and PC3 cells were incubated with the 100µl RPMI-1640 media and 100µl

conditioned media prepared by incubating the isolated bacteria into RPMI-1640 media overnight and cytotoxicity was determined as described in Materials and Methods. *P* values were determined using two sample T- test, two- tailed distribution. The results represent mean ± standard error of several experiments performed in duplicate. **B: Representative images of the cytotoxic effect of tiger prawn conditioned media against HaCaT, HeLa and PC3 cells.** For the representative images, an inverted light microscope was used to capture images of the cells in each well at x200 magnification. [CM1: *A. baumannii*, CM2: *P. luteola,* CM3: *P. oryzihabitans*, CM4: *S. xylosus*, K12: *E. coli* K12, N: negative and P: positive]. The results are representative of several experiments. **C: The trypan blue staining images for the cytotoxic effect of tiger prawn conditioned media against HaCaT, HeLa and PC3 cells.** The cells from LDH assays were subjected to fixation using methanol and acetone in a ratio of 1:1, followed by cell viability staining using trypan blue. [CM1: *A. baumannii*, CM2: *P. luteola,* CM3: *P. oryzihabitans*, CM4: *S. xylosus*, K12: *E. coli* K12, N: negative and P: positive]. The results are representative of several experiments.

2.3. Centipede and prawn lysates showed anticancer properties activity against HeLa and HaCaT cells

Cytotoxicity assays were performed to determine the cytotoxic effects of prawn's lysates against HaCaT, PC3 and HeLa cells. As shown in Table 4, the prawn gut showed 20% and 8.6% cytotoxicity against Hela and PC3 cells but no cytotoxic activity against HaCaT cells were observed. These are remarkable findings and clearly show that selected organ lysates possess anticancer molecules. Importantly, centipede showed 30% and 72% cytotoxicity against Hela and PC3 cells but no cytotoxic effects were observed against normal HaCat cells (Table 4). The organ lysates showed broad-spectrum anticancer properties (Table 4). In growth inhibition, cricket gut lysates and dubia cockroach inhibited growth of PC3 cells compared with HaCat cells (Table 4).

Table 4. Effects of lysate of selected animals on growth/cell death of human cancer and normal cells.

Invertebrates		% Growth			% Cell Death		
		HeLa	PC3	HaCaT	HeLa	PC3	HaCaT
Control			**100%**			**0%**	
Scolpendra subspinipes (centipede)	Head	17.8±12.1	0±0	0±10.6	13±12.5	69.2±26.6	45±27.0
	Reproductive system	35.1±4.1	0.5±0.7	21.5±7.6	1±1.0	0±0	19±18.5
	Body	15.6±13.1	50.4±2.0	12.0±39.8	35±17.0	9.4±0.7	13±13.0
	Egg	51.6±0.5	65.2±0.3	0±4.0	0±0	0±0	0±0
	Haemolymph	0±1.8	0±0	0±8.7	30±1.0	72.7±27.3	5±4.5
Penaeus monodon (tiger prawn)	Gut	15.3±19.0	0±0.4	0±46.3	8.6±2.3	20.4±18.9	0±0
	Eye	100±17.2	100±3.3	40.3±12.0	9.9±3.8	0±0	0±0
	Exoskeleton	100±16.5	94.9±14.1	19.4±1.4	72.1±3.7	0±0	0±0
	Body	61.3±9.3	70.1±2.6	33.6±39.6	57±6.0	0±0	48±16.5
	Head	85.2±14.3	94.8±11.0	81.5±15.9	13±18.4	0.9±0.9	19±19.0
	Appendages	100±6.2	82.1±2.5	100±64.2	0±0	11.3±2.9	20.5±14.5
Acheta domesticus (cricket)	Head	69.7±15.1	45.1±2.3	58.2±16.7	0±0	0±0	0±0
	Gut	63.5±19.6	22.3±0.7	45.5±12.1	28±16.0	0±0	10±10
	Upper abdomen	62.4±1.5	32.2±5.0	28.6±14.6	6±6.0	57±43	32±21
Blaptica dubia (dubia roach)	Egg	62.5±2.6	50.1±4.1	74.8±21.2	18.0±4.5	0±0	0±0
	Gut	100±3.4	100±15.2	33.5±18.9	0±0	0±0	6±5.5
	Upper abdomen	79.3±2.4	47.1±4.5	42.7±14.9	0±0	0±0	0±0

3. DISCUSSION

This study focuses on identifying potential anticancer agents in selected invertebrates and their gut microbiota. Several invertebrates were selected due to the natural polluted habitats as shown in Table 1. Although, recent studies have tested human gut microbiota for anticancer agents [14,15], to our knowledge, this is the first study to mine gut bacteria of selected invertebrates for potential anticancer molecules. Zhou et al., [15] revealed the anticancer activity of gut bacteria isolated from the fecal sample of a group of healthy individuals; preschool children and university students. If bacteria isolated from humans can possess anticancer properties, it is logical to speculate that pests that thrive in polluted environments and are able to withstand high levels of radiation may contribute to the resistance of cancer development in the host species [13,16]. Notably, *Homo sapiens* are just one species among millions of other species and we are a relatively new

addition to this planet. Other species such as cockroaches, crocodile/alligator have shown the ability to adapt, evolve and survive successfully over millions of years, suggesting that we ought to learn from these species [12,13]. Besides their immunity, their gut microbiota likely contribute to their protection against communicable and non-communicable diseases by secretion of antimicrobial and anticancer agents respectively. In this regard, the microbial world has attracted increasing attention due to its' ability to thrive in different environments by synthesizing bioactive molecules. The by-products synthesized by bacteria are becoming more valuable in the medicinal field. Studies have been conducted to examine the anti-cancer properties of microorganisms isolated from various environments; water, plant extracts, soil and clinical samples. Phonnok et al., [17] reported the anti-cancer activity of a spectrum of bacteria including *A. baumannii, Pseudomonas aeruginosa* and *Bacillus* sp., which exhibited cytotoxic effect against HeLa cervical cancer cells leaving normal cell line (Vero) unaffected. The study also indicated that the extracts of those bacterial species inhibited the growth of HeLa cells without having any significant effect on the normal cells [17]. The anticancer activity of five strains of soil microorganisms isolated from Tangkuban Perahu mountain against T47D breast cancer cell line were demonstrated. Our findings are consistent with these studies and clearly showed that bacteria isolated from novel sources such as polluted environments are potential sources of anticancer molecule(s).

In this study, conditioned media of the invertebrates' aerobic gut microbiota were prepared and tested for anticancer activity. The results revealed that CM3 from tiger prawn corresponding to *P. oryzihabitanss* and CM1 from centipede corresponding to *K. varians* exhibited cytotoxic effects and growth inhibitory effects against both HeLa and PC3 cells. However, the identity of the active molecules remains unknown and it is the subject of future studies. Of interest, Karpiński and Szkaradkiewicz [18] identified several anticancer peptides from bacteria including azurin, a 14 kDa peptide synthesized by *Pseudomonas* that was shown to trigger apoptosis in tumor cells by inducing the activation of the caspase cascade [19]. Beside from forcing cells to undergo apoptosis, this peptide exhibited penetration into tumor cells compared with the heathy cells. Moreover, antibacterials such as actinomycin D, bleomycin, doxorubicin and mitomycin C synthesized by bacteria exhibited anticancer properties [20]. The anticancer mechanisms of each of these molecules are however different from each other. While actinomycin D triggers p53-independent apoptosis in cells [21] and bleomycin exhibit oxygen and metal ion-dependent cleavage of the DNA [22]. Doxorubicin, in turn, inhibits DNA and RNA replication and transcription and causes oxidative stress in tumor cells, triggering membrane, protein and DNA damage [23]. Similar mechanisms may explain our findings. In conclusion, these results show that the gut microbiota of selected invertebrates produce molecules with anticancer properties. Thus, these molecules could potentially be used as drug leads for the rational development of therapeutic agents against cancer cells, however, intensive research over the next few decades is needed to realize these expectations.

This study also shows that cherry red centipede organ extracts and serum, tiger prawn organ extracts, and house cricket organ extracts affect the viability of cancerous cell lines, HeLa and PC3. This novel finding is highly significant as it may lead to the identification of novel compounds that may be able to target and destroy cancer cells. Although the actual molecules that act against cancer cells and their mechanism of actions are yet to be determined, it is obvious that tiger prawn body and gut, cherry red centipede head, body, reproductive system, egg and haemolymph, and house cricket upper abdomen and gut are the organ extracts that contain molecules that can act against cancer cells.

Our results are consistent with previous studies such as a report where it was shown that compounds from *Penaeus latisculatus* (king prawn) showed anticancer activity [24] and another report where it was shown that red cherry centipede lysates exhibited anti-cancer activity when tested in mice infected with S180 sarcoma and H22 hepatoma [25]. Interestingly, lysates such as the tiger prawn head and gut, cherry red centipede haemolymph, and house cricket gut showed cytotoxic effect against cancer cells but had negligible or no cytotoxic activity against normal cells

4. CONCLUSIONS

In conclusion, we showed that the organ lysates and gut bacteria of selected invertebrates residing in polluted environments exhibit potent anti-tumour activity. These findings further suggest that animals residing in polluted milieus are a large unexploited source for prospective pharmaceutical drugs that may lead to the identification of novel anti-tumour compound(s) and/or understanding of the mechanisms of cancer resistance in such species, however extensive research over the next few years is needed to realize these expectations.

5. MATERIALS AND METHODS

5.1. Dissection

Acheta domesticus (cricket), *Anadara granosa* (blood clam), *Blaptica dubia* (cockroach), *Penaeus monodon* (tiger prawn) and *Scolpendra subspinipes* (centipede) were procured (Table 1). Various organs were extracted, and samples collected as described previously [12,13,26,27] (also shown in Fig. 1). All the organs were kept on ice during the dissection. Once extracted, the organs were homogenized in the presence of protease inhibitors and EDTA. Next, lysates were freeze-thawed ten times and sonicated at 30GHz. The samples were then centrifuged at 15000 x g for 80 min at 4°C. The supernatants, known as lysates, were collected and filtered using filters of 0.2 µM 0.2 pore-size and stored at -80°C (Siddiqui et al., 2017). The Bradford assay was used to determine the concentration of protein in the lysates and tested for against human cancer and normal cells.

To isolate gut bacteria of invertebrate species, *A. granosa* shell was opened using a pair of scissors, and the middle section of the invertebrate was dissected to expose the gut. Next, bacteria were isolated from the gut using a sterile cotton swab and streaked on blood agar as well as nutrient agar plates. For *A. domesticus, B. dubia, P. monodon* and *S. subspinipes*, invertebrates were deactivated by incubating on ice for 5 min and the dissection was carried out as described above. The agar plates were then incubated overnight at 37^{o}C (Akbar et al., 2018). Following the incubation, bacterial colonies were isolated based on their texture, size, color and shape onto fresh blood and nutrient agar plates. The bacterial colonies were then subjected to identification through Analytical profile index (API) [26,27].

5.2. Analytical profile index (API) identification

Prior to API identification, Gram staining was conducted. API staph was used for the identification of Gram-positive and catalase positive bacteria, while API 20E was used for Gram-negative and oxidase negative bacteria. API identification was conducted by inoculating the bacterial species into the respective API followed by 18 to 24 h incubation at 37^{o}C. After incubation, additional tests were conducted, and the results were recorded to determine the identity of the bacteria.

5.3. Preparation of bacterial conditioned medium

Conditioned media were prepared by inoculating single colonies of bacteria in RPMI-1640 medium, followed by 24 h incubation at 37^{o}C in an aerobic environment with shaking. The cultures were subjected to centrifugation at 4^{o}C, 12,000 x g, for 1 h. Next, the supernatants were collected and filtered using a 0.22 µm pore size syringe filter. The conditioned media were then stored at -80^{o}C [26,27]. Bradford assay was conducted to determine the protein concentration of the conditioned media.

5.4. Cell cultures

Cancer cell lines; HeLa (ATCC® CCL2™) and PC3 (ATCC® CRL1435™) and normal cell line; HaCaT; acquired from American Type of Culture Collection, were used in this study. All the cell lines were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin streptomycin antibiotic and 1% Minimum Essential Media (MEM) Non-Essential amino acid at 37^{o}C, with a supply of 5% carbon dioxide and 95% humidity [12,13].

5.5. Cytotoxicity assays

Lactate dehydrogenase (LDH) assay was initiated by seeding the cells (HaCaT, HeLa and PC3) and incubated at 37^{o}C with 5% CO_2 and 95% humidity. LDH was conducted using confluent cell monolayers as described previously (Siddiqui et al., 2017; Akbar et al., 2018). Briefly, cells were incubated with organ lysates, conditioned media and RPMI-1640 media for 24 h at 37^{o}C with 5% CO_2 and 95% humidity. After incubation, the supernatants were collected and the percentage cytotoxicity was calculated as follows: % cytotoxicity = ((Absorbance$_{sample}$ – Absorbance$_{negative\ control}$)/ (Absorbance$_{positive\ control}$ – Absorbance$_{negative\ control}$)) *100, whereby the negative control consist of cells treated with conditioned media from non-pathogenic *E. coli* K-12 bacteria and RPMI-1640 media only, while the positive control consisted of cells treated with Triton x-100

This assay is based on measuring lactate dehydrogenase (LDH) release; a soluble cytosolic enzyme, found in all cells, released into the culture medium by damaged cells only. The principle of this assay is that cell supernatant containing LDH catalyses the conversion of lactate (solution from kit) to pyruvate, generating NADH and H+. In the second step, the catalyst (diaphorase, solution from kit) transfers H and H+ from NADH and H+ to the tetrazolium salt p-iodo-nitrotetrazolium violet (INT), which is reduced to formazon (dye), and absorbance is read at 492 nm. The cells incubated alone were used as negative controls, whereas monolayers

lysed with 1% Triton X-100 for 30 min at 37°C were used as 100% cell death. Control values were acquired from cells incubated alone and total LDH release was measured from cells treated with 5% Triton X-100 for 1 h at 37°C. To further determine the viability of treated cells, survival assays were performed. Briefly, cells treated with lysates and conditioned media were collected and seeded onto new plates containing growth media.

5.6. Growth inhibition assays

Assays were performed to determine the effects of organ lysate(s) and conditioned media on cell growth. Briefly, cancer cells and normal cells were inoculated in 96-well plates for 24 h at 37°C in a 5% CO2 incubator until a semi-confluent monolayer (up to 50% confluency) was formed. At this stage, cells were trypsinized and cell count was determined using a haemocytometer. Next, organ lysates and conditioned media were added to the semi-confluent monolayer of cells and plates were incubated for 24h at 37°C in a 5% CO2 incubator. For controls, cells were incubated with complete growth medium and/or BSA. Following this incubation, the cells were trypsinized with 2.5% trypsin for 15 min and enumerated using a haemocytometer.

5.7. Statistical analysis

Statistical significance for differences was evaluated using a 2-sample t-test; two-tailed distribution, comparing the mean of two different experiments repeated using similar conditions. *P* values <0.5, <0.1 and <0.05 were used for analysis. For graphical representation of the data, y-axis error indicated the standard error of the data between the repeats on the figure.

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