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Comparative analysis of the antibacterial, antifungal, antiproliferative and cyclic response element (CRE) induced expression of downstream luc gene activities of *Monopterus albus* and *Channa straitus* extracts

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ABSTRACT

In Malaysia, *Monopterus albus* is commonly found in rice fields, muddy ponds and swamp areas. *Channa striatus* have been widely used as a source of traditional medicines. The extracts of Malaysian local *Monopterus albus* and *Channa straitus* have been reported to have different bioactive properties and these properties can be used at molecular level as alternative tool for different disorders. The comparative analysis of antibacterial, antifungal, antiproliferative and CRE induced expression of downstream *luc* gene activities of both the extracts were performed. The bacteriostatic and antibacterial effects of both extracts were revealed beside higher antifungal activity of *Channa straitus*. The extracts from *Monopterus albus* showed higher levels of antiproliferative activity as compare to *Channastraitus*. The results were found supportive towards up regulation of *hrluc* by *Monopterus albus* extracts and down regulation by *Channa striatus* extracts. This is the first report on comparing the bioactive properties of *Monopterus albus* and *Channa straitus*. The results from this study demands a further research on identifying the bioactive molecules involve in these actions at a molecular level.

INTRODUCTION

Country side has been and still is a incessant cradle of medicinal products. When saying this, many scientists might only think at herbal vegetation as spring of medicinal bioactive. In point of fact, animals are a so far ill-reconnoitred source for medicines even though they are well known ingredients for many prevalent medicines, some of them documented by current and/or past pharmacopoeias around the world. Snakes, frogs and other various insects are used in many Asitic Materia Medica; Spanish flies and leeches were listed for a long time in Western Pharmacopoeias and maggots has been recently listed in the US Pharmacopoeia (Root-Berstein and Root Bernstein, 1999; Rubin, 2004). In the preceding epochs, a prodigious devotion has been paid to marine animals mainly sponges, but non-marine animal drugs are still principally derelicted by researchers as a source of medicines perhaps because they pose somber complications including complex chemical matrixes, reduced yield on bioactive, ethical glitches, protection by the authorities and sometimes difficulty in finding a reliable and viable supply. Malaysia is, outside any doubt, gifted with profligate flora and fauna species providing to its inhabitants with a exceptional source of stable foods as well as medicinal products.

Native medicinal plants have been a very popular health choice among Malaysians but the traditional knowledge also includes the medicinal use of animal drugs, such as fishes, insects and others. The Malaysian local swamp eel (*Monopterus albus*) and snakehead fish (*Channa striatus*) are traditionally consumed as part of local delicacies. It is also known that eels and snakehead fish have been widely used as a source of traditional medicines such as the cure for kidney disease and impotency as well as hastening healing of surgical wounds. Haruan (*Channa striatus*) is in great demand in the Malaysian domestic fish market.

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Detailed knowledge of the genetic diversity and population genetics of C. striatus is needed for sound management, conservation, stock identification, and successful farming of the species. Haruan, the local name for the snakehead C. striatus, is an obligate freshwater fish of the family Channidae that has important economic value as a food fish, and has pharmacological properties as well as medicinal value (Mat Jais, 1991, 2007a, 2007b; Rahim et al., 2009; Jamaluddin et al., 2011).The wound healing process can be delayed by infection of micro-organisms that are normally sequestered at the skin surface which had obtained access to the underlying tissues (Edwards and Harding, 2004).Various studies had been done with the intention to investigate potential healing process of eels and snakehead fish. Previous study had shown that eels and snakehead fish had the ability to provide antimicrobial and antifungal effect. (Bragadeeswaran and Tnangaraj, 2011, Haniffa *et al*., 2009). Both were found to have a potential contribution in assisting the process of wound healing. (Pravin Kumar *et al*., 2012, Nik Mohd Ikram and Ridzwan, 2013). Even though there are studies performed on the effect of eel and snakehead fish alone, there is none involved in comparing both of the species. We thus aim to compare the effect of anti bacterial, anti fungal effect and in addition anti proliferation as well as gene expression effects of the both eel and snakehead fish extracts.

METHODS

Preparation of *Monopterus albus* and *Channa striatus* Extracts

The extracts were prepared by the method described previously (Uthayakumar *et al*., 2012) with slight modifications. The fishes were acclimatized to artificial cave fitted tank and after the organisms were sacrificed, the flesh along with the skin was cut into small pieces and then meshed to lyophilize. There were two extracts prepared. Extract A: The lyophilized samples of eel and snake head fish (1mg/ml) were suspended in 95% ethanol for 3 times. The ethanol extract was pooled evaporated and suspended in distilled water to get a final volume of 50 ml and extracted with dichloromethane (Ch_2Cl_2). Extract B: The lypholized extracts (Extract A) were dissolved in distilled water and 5% dimethylsulfoxide.

Antibacterial activities of the extracts

The antibacterial activities of the extracts were determined against two bacterial human pathogens; *E.coli* and *Vibrio cholera* by the methods described previously (Uthayakumar *et al*., 2012). This was determined by using standard diffusion disc plate assay (Lehrer *et al*., 1991) by determining the suppression zone of bacterial growth around 3mm diameter well measured in millimeters.

Antifungal activities of the extracts

The antifungal activity of the of the extracts were determined by measuring inhibition zone after 24 hours incubation on potato dextrose agar medium adjusted with McFarland density to obtain final concentration of 10^4 CFU/ml. The diluted extracts (5%) were applied on each fungal strain medium and were incubated at 28^{o}C. Four strains were used for this purpose which includes Aspergillusniger, Aspergillusflavus, Candida albicans and Mucor species. Standard antibiotics were included as antifungal control as used previously (Uthayakumar et al., 2012).

Generation time and growth rate of *E. coli and V. cholerae*

A final concentration of *E.coli* was obtained by diluting an overnight culture in 10 ml of LB broth. The bottles were incubated in shaker at 200 rpm at 37^{o}C. The culture's OD was measured at 600 nm, after every hour post-incubated. A line graph was obtained by plotting the OD_{600nm} against the calculated CFU.*E. coli* bacteria were maintained on non-selective LB agar plates and were harvested in non selective LB broth. Vibrio cholerae were maintained in LP broth supplemented with polymyxin B (0.75µg/ml). For the preparation of growth curves, final concentrations of *E. coli* were obtained by diluting overnight cultures of in 10 ml of LB broth. The bottles were incubated at shaking condition (200 rpm) at 37^{o}C. The OD of the culture was measured after every hour post-incubated at 600 nm. A line graph was obtained by plotting the OD_{600nm} against the calculated CFU. For the growth curves of *V. cholera,* overnight culture of *V. cholerae* was diluted in 10ml of LB broth with appropriate supplement (0.75 µg/ml Polymyxin B) so that final concentration was $1x10^6$cells/ml.

The culture was incubated at 37^{o}C while shaking at 200 rpm. An aliquot of the culture was taken every 1h and its OD was measured at 600 nm. Growth curves were shown as line graphs where number of cells was plotted against OD_{600} nm. The growth rate (K) was calculated by using the formula (K=*log* 10 X*t* – log 10 X*o*/0.301 x *t.* The generation time (t_{gen})was calculated from the calculated value of growth rate(K) by using the formula (t_{gen} = 1/*K*).

Antiproliferative activity of extracts

HeLa cells were seeded into 96-well microtitre plates (Becton Dickinson Franklin Lakes, NJ) at 2.5 x 10^5 cells/well in 100 µl of DMEM with 10% (v/v) of fetal bovine serum at 5% CO2 concentration. The cells were treated for total of three days with all the four extracts beside a negative untreated HeLa cells lines. The cells were treated on second and third day. At day four, cell viability was measured by conventional MTT assay. A total of 50µl of 5mg/ml of MTT reagent in PBS was added to all treated and untreated HeLa cells' wells.

To this, a total of 150 µl of DMSO was added to each well. This resulted in solublization of invisible purple formazine precipitated which was produced by the reduction of MTT reagent by the viable HeLa cells. This formazine dye was measured at 570 nm. All assays were performed in triplicate. The cytotoxic effect in MTT assay is defined as;

$$[(A_{570\ nm} \text{ of treated cells}/ A_{570} \text{ nm of untreated cells})] \times 100.$$

Maintenance of HeLa cell culture and sub-culturing

The HeLa cells were thawed by shifting the vials from nitrogen tank to a water bath at 37°C for 2 minutes. During the warming, the vial's cap was loose to avoid minor explosion by reducing the pressure in vial. The cells were suspended in total of 5 ml of medium and were transferred to 15 ml sterile falcon tube. The tube was centrifuged at 1,000 rpm for 5 minutes and supernatant was removed. The cell pallet was resuspended in 15 ml of medium. The cell suspension was transferred to a sterile culture flask to be incubated at 37°C in a humidified atmosphere of 5% CO_2 for 24 hours in a CO_2 incubator. The medium was changed every three days (or sometimes depending on the color of medium). The confluency of the cells was observed and the medium was discarded. Using the sterile PBS the cells were washed in order to remove any excessive medium or dead cells from the flask. A total of 2 ml of 5% (v/v) Trypsin-EDTA solution was poured into 25 ml culture flask and the flask was transferred to 37°C for 5 minutes. The neutralization of trypsin was performed by adding 2 ml of complete medium followed by transfer of suspension into a sterile 15 ml falcon tube. This suspension was then subjected to centrifugation at 1,000 rpm for 5 minutes at room temperature followed by discarding the supernatant. The re-suspension of cell pallet was done by adding 5 ml of pre-warmed medium followed by cell counting using improved Neubauer. The appropriate volume of medium was added and the cell suspension was transferred into a new sterile flask to be incubated in CO_2 incubator.

Co-transfection of reporter gene vectors into HeLa cell lines and Luciferase assays

Transfection of the reporter and control vectors were performed using the commercially available TransFast™ (Promega) kit. The transfection into HeLa cell lines was performed in 6 wells plate. The plating cell density for 6 wells plate was calculated to be 2.5 x 10^5 cells/ml (growth area for 6 wells plate was 9.2 cm^2 and the relative area was 5X). The luciferase assay was performed with Dual Luciferase® Reporter Assay System (Promega) and luminescence was measured by GloMax® 20/20 Luminometer (Promega). Two test reporter gene vectors; pGL4.74 (h*Rluc* test reporter gene; Invitogen Corp, USA) and pGL4.74::CRE (pGL4.74 with a 720 bp from proximal promoter of human survival of motor neuron gene with a cyclic response element (CRE) inserted at *Eco*RI and *Hind* III in pGL4.74) were used with a control vector pGL4.10 (*luc*2 control reporter gene; Invitogen Corp, USA) for the co-transfection into HeLa cell lines with the optimization charged ratio of charge ratio of 1:2 of 2 µg/ml pGL4.74 to TransFast which was confirmed. The post transfection incubation time was optimized by transfecting 2 µg/ml pGL4.74 and pGL4.10 (2 µg/ml) into HeLa cell lines and it was determined to be significant (0.731) at two hours (Pearson correlation (r) = -0.213). The RLU values of all the samples were obtained and were normalized for data correction to be presented as Ratio of RLU of reporter (h*Rluc*) to control gene (*luc*2) (R/F), Normalized fold change (in activity) of h*Rluc* / *luc*2 for different extracts and percent activity of luciferase determined in different extracts.

RESULTS

Antibacterial activities of the extracts

The results of antibacterial activity (Table 1) reflects a significant comparative difference (63.6 % and 83.3%) between the restriction zones for extracts (A and B) from *Monopterus albus* which were higher from the restriction zones observed and measured for *Channa striatus* extracts (A and B) respectively. The restriction zones were found to be higher as compared with the restriction zones observed in ampicillin as a control (5 $\pm$ 2 for *E.coli* and 7$\pm$3 for *V. cholerae*). The growth curves plotted for *E.coli* and *V. cholerae*against *Monopterus albus* and *Channa straitus* extracts was used to calculate the generation time and growth rate of these pathogens and calculations showed the bacteriostatic effect of these extracts (Table 1).

The generation time and growth rate for *E.coli* and *V. cholerae* were found higher in extracts of *Monopterus albus* as compared to *Channa straitus* (Table 1) with the extract B in both cases to show higher values of generation time and growth rate. These results reflect the bacteriostatic and antibacterial effect of *Monopterus albus* and *Channastraitus* extracts.

Anifungal activities of the extracts

All the extracts showed higher anti fungal activity as compared to the control (ampicillin). The extract A (ethanol) from *Channa striatus* showed maximum antifungal activity as compared to other abstracts (table 2).

Antiproliferative activity of extracts

The cytotoxicity for all the extracts were measured for HeLa cell lines and maximum toxicity was found for extracts A (ethanol) for *Monopterus albus* (45%) and *Channa striatus* (51 %) as compared to the extracts B (aqueous) for *Monopterus albus* (58 %) and *Channa striatus* (67 %). Overall, the extracts from *Monopterus albus* was found higher (11.76 % and 13.43 % for extract A and B respectively) than extracts from *Channa striatus*. The extracts from both animals showed anti proliferative activity with higher activity for *Monopterus albus* (figure 1).

Effects of extracts on gene expression

The mean *hrluc*/*luc*2 for for *Monopterus albus* and Channastraitus treated HeLa cells transfected with pGL4.74 parental vector (with *hrluc*) and pGL4.74::Pro720 (with CRE site) was measured. The cotransfection of pGL4.10 (with *luc*2) was performed for the normalization of data for luciferase assay. It was found that the mean RLU for pGL4.74 and pGL4.74::CRE constructs treated with *Monopterus albus*extracts were higher (76.4% and 67.8%) respectively as compared to the mean RLU for pGL4.74 and pGL4.74::CRE constructs treated with *Channa straitus* extracts. The untreated pGL4.74 was used as a negative control for luciferase assay beside linearized pGL4.74 as a

negative control for co-transfection. The results suggested the down regulation of downstream *hrluc* gene by *Channa straitus* extract but upregulation of *hrluc gene* by *Monopterus albus*. Percent activity of luciferase and was calculated as a process of normalization of data for luciferase assay and the results were found consistent with the mean RLU values. The percent activity of luciferase for all the constructs showed to be highest for the transfections of pGL4.74 and pGL4.74::Pro720 (76.4% and 67.8%), which were treated with *Monopterus albus* extract as compared to the *Channa striatus* extracts (figure 2). The results were found supportive towards up regulation of *hrluc* by *Monopterus albus* extract and down regulation by *Channa striatus* extracts. The difference in luciferase activity was calculated to be maximum for 1.07 (95.32 % of pGL4.74::Pro720 treated with *Monopterus albus* extract, 85% of pGL4.74::Pro720 treated with *Channa striatus* extract, 95.32 % of pGL4.74 treated with *Monopterus albus* extract and 86.91% of pGL4.74 treated with *Channa striatus* extract). The luciferase activity of pGL4.74:: Pro720 treated with *Monopterus albus* was found higher (14%) than the luciferase activity of pGL4.74:: Pro720 treated with *Channa striatus* (85.98% of *Monopterus albus* treated). Similarly, for the pGL4.74 without CRE site, the luciferase activity of pGL4.74 treated with *Monopterus albus* was found higher (9%) than the luciferase activity of pGL4.74treated with *Channa striatus* (91% of *Monopterus albus* treated). The results of luciferase assays were in accordance with the normalized values and were found significant by Pearson correlation analysis.

Table 1: Antibacterial activity of *Monopterus albus* extracts (2mg/ml) and *Channastraitus* extracts (2mg/ml)against *E.coli* and *V. cholera* (mean ± SD).

Pathogens		*Monopterus albus* extracts		*Channa staraitus* extracts		Untreated
		Extract A	Extract B	Extract A	Extract B	
E. coli	Antibacterial activity	11.00 ± 1	12.00 ± 2.00	7.00 ± 2.00	10.00 ± 2.00	Mixed growth
	Growth rate (generation/hour)	5.5	4.7	6.6	5.8	3.57
	Generation time (Hours/generation)	0.18	0.21	0.15	0.17	0.28
V. cholerae	Antibacterial activity	11.00 ± 1	13.00 ± 1.00	8.00 ± 3.00	10.00 ± 1.00	Mixed growth
	Growth rate (generation/hour)	4.3	3.8	5.2	4.7	3.31
	Generation time (Hours/generation)	0.23	0.26	0.19	0.21	0.30

Table 2: Antifungal activity of *Monopterus albus* extracts (2mg/ml) and *Channastraitus* extracts (2mg/ml)against *Aspergillusniger*, *Aspergillusflavus*, *Candida albicans*, *Mucorsp* (mean± SD).

Fungal Pathogens	*Channa staraitus* Extracts		Eel Extracts	
	Extract A	Extract B	Extract A	Extract B
Aspergillus niger	7.00 ± 0.8	6.80± 0.5	8.20 ± 1.00	9.00 ± 1.00
Aspergillus flavus	7.80 ± 0.6	8.20 ± 0.6	9.30 ± 0.5	9.80 ± 0.55
Candida albicans	9.00 + 2.00	11.00 + 1.00	8.00 + 1.00	7.25 + 0.5
Mucor sp	8.00 + 0.8	10.20 + 2.00	8.55 + 1.00	9.20 + 0.55

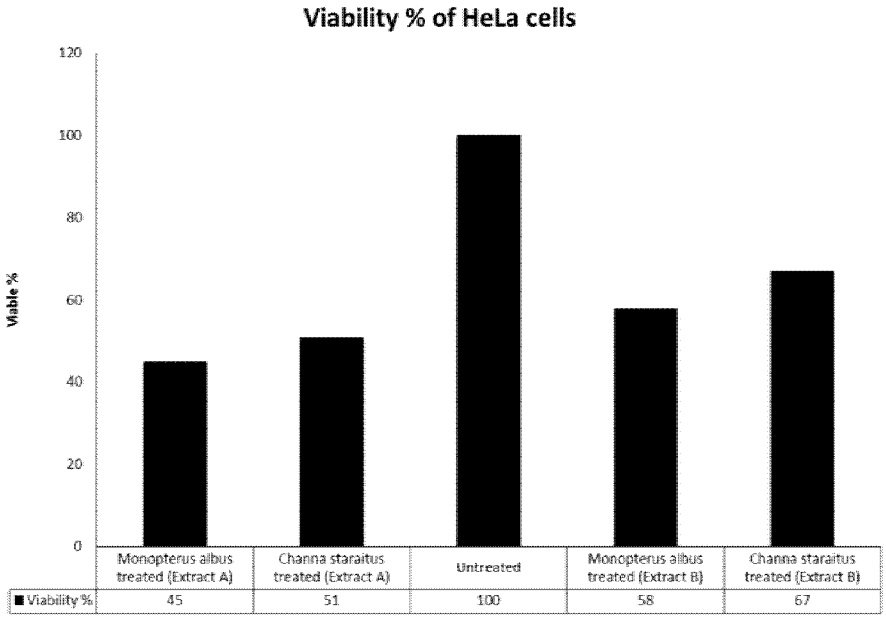

Fig. 1: Effects of treated and untreated both *Monopterus albus* and *Channastraitus* extracts on cancer cell line (*HeLa* cells).

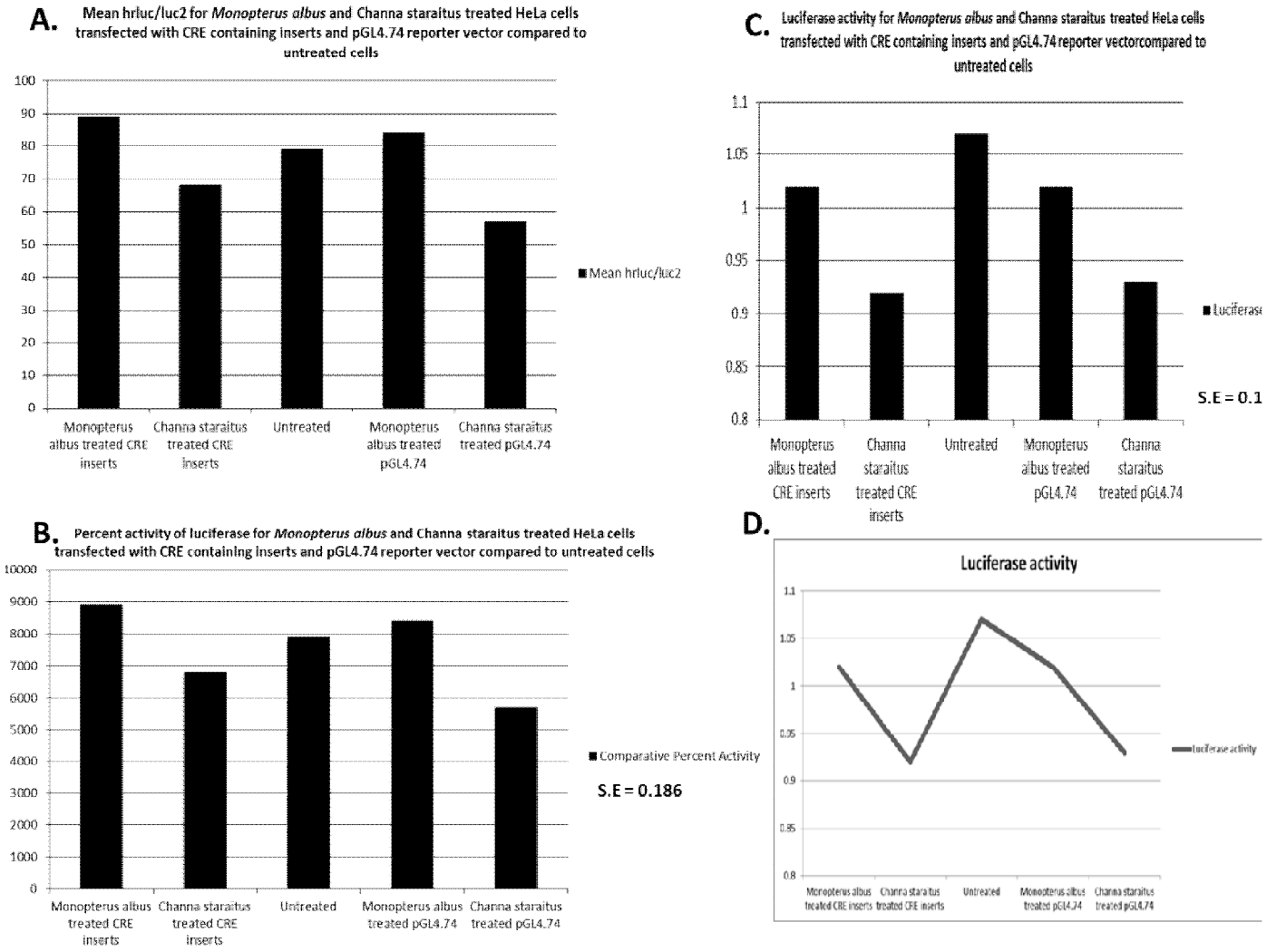

Fig. 2: Effects of extracts from *Monopterus albus* and *Channa straitus* on treated HeLa cells transfected with CRE containing inserts and pGL4.74 reporter vector compared to untreated cells.

DISCUSSION

The role of *Channa striatus* and *Monopterus albus* in wound healing, antibacterial activities, antifungal activities has been accepted traditionally in Malaysia and neighboring countries with strong scientific evidences with *Channa striatus* being indigenous to many of such tropical countries (Mohsin *et al*., 1983). The flesh is claimed in rejuvenating in recuperation from serious illness and in postnatal diet beside the use in wound healing (Mat Jais *et al*., 1994). Many claims have been considered about the flesh of both animals without any scientific claims. We planned to screen and identify the bioactive molecules in the flesh of these two animals. Instead of targeting a specific organ or component, we aimed in using the whole animal to avoid any loss of any crucial element in future analysis of bioactive molecules by UHPC and LCMS etc. This study was a preliminary study to compare the effects of *Channa striatus* and *Monopterus albus*. The results showed that the antimicrobial effects (anti bacterial and anti fungal) of extracts from both animals were markedly high as compare to control (ampicillin) but if, we compare the antibacterial and antifungal effects of both the animals, extracts from *Monopterus albus* showed higher antibacterial and antifungal activities (table 1 and 2).

The antiproliferative effect on HeLa cell lines reflected again that both the extracts have antiproliferative (by MTT assay) activity but again the *Monopterus albus* showed a little more activity (11.76 % and 13.43 % for extract A and B respectively)than *Channa striatus* (figure 1).Previous studies on the antiproliferative activity of fish protein hydrolysates on human breast cancer cell lines also supports this present piece of work (L. Picot *et al*., 2006).For the future plans, as we are looking into the role of these extracts at molecular level, the effects of these effects were analyzed on the gene expression. We used pGL4.74 vector which has an insert of *hrluc* in its backbone. Besides *hrluc*, we cloned a region from human promoter (promoter region of *SMN*2 gene in humans) with a CRE binding site as an enhancer. We choose the region with CRE binding site as it is one of the cAMP response element involved in transcriptional control of many of the human genes (Sarmila *et al*., 2003). The results of co-transfection were interesting because likewise in antibacterial and

antifungal, where *Monopterus albus* was more effective, in gene expression analysis, again the aqueous extract from *Monopterus albus* was found more effective in over expression of downstream gene (*hrluc*) in the presence and absence of human promoter region (figure 1 and 2). The results were optimized to normalize the data (table 3). Interestingly, *Channa striatus* was found to down regulate (decrease in expression) the expression of downstream gene (*hrluc*) in the presence and absence of human promoter region (figure 1 and 2). These all findings reflect the more dominant role of extracts from *Monopterus albus*over *Channa striatus*. These findings also suggest, if the role of *Channa striatus* in down regulating the expression of the gene is well understood using microarray expression analysis then, might the extracts or bioactive molecules from *Channa striatus* can be used as a therapy at molecular level. Similarly, the molecular analysis of the *Monopterus albus* in future can help to find some novel therapies for up regulating the human genes. He Y et al did report the presence of biochemical macromolecules or expression analysis in *Channa striatus* (He *et al.*, 2006) and *Monopterus albus* but yet, no comparison has been reported among biological activities of extracts from both of these animals. To the best of our knowledge, this is the first preliminary study on the comparative analysis of the extracts from *Monopterus albus* and *Channa straitus*.

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