Journal of Zankoy Sulaimani
Part-A- (Pure and Applied Sciences)
http://jzs.univsul.edu.iq

In vitro Cytotoxic Activity of Some Fecal Filtrates

Karokh Ali Khdir[1*] & Bahrouz Mahmood Al-Jaff[1]

[1]*Biology Department, College of Education, University of Sulaimani, Sulaimani, Kurdistan Region- Iraq*
* *Corresponding author's e-mail*: karokh.khdir@univsul.edu.iq

Article info

Original: 3 January 2020
Revised: 25 February 2020
Accepted: 5 April 2020
Published online: 20 June 2020

Key Words:

Fecal filtrate
Mixed fermentation
Cytotoxic activity
Cervical cancer cells

Abstract

Animal feces have been studied and recognized as a crucial resource for exploring and discovering new novel bioactive compounds produced by host, microbiota, or host-microbiota interaction that may have therapeutic importance. To investigate the cytotoxic effect of human (healthy and colorectal cancer), dog, and cow fecal filtrates that serves as natural bioreactors. The cytotoxic activity was calculated as inhibitory concentration (IC50) based on the percentage of % viability using MTT 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay for 4 crude cell-free fecal filtrates and their Sephadex G100 fractions in vitro against HeLa human cervical cancer cell line. The optical densities (OD) of the fractions were checked at wavelength of 280 nm and considered in the assay rather than cytotoxic active compound concentrations. Cytotoxic activity of each crude fecal filtrate appeared to be dose-dependent ($P<0.001$) and less active than 40-400 µg/ml 5-flourouracil (5-FU). IC50 for dog, cow, healthy human, colorectal fecal filtrates, and 5-FU were 442.64 ± 23.29, 1265 ± 35.8, 1715 ± 56.9, 400.76 ± 32 and 134.33 ± 3.29 µg/ml respectively. Out of 11 dog fecal filtrate fractions, 4 fractions (F4, F5, F6 and F7) were within IC50 range. Out of 10 cow fecal filtrate fractions, 3 fractions (F3, F5 and F6) were within IC50 range. Out of 11 healthy human fecal filtrate fractions, 2 fractions (F3 and F4) were within IC50 range. Out of 12 colorectal fecal filtrate fractions; 4 fractions (F2, F3, F4 and F6) were within IC50 range against HeLa cells. The crude fecal filtrates and their fractions were with apparent cytotoxic activity showed that the colorectal patients and dogs' fecal filtrates have higher cytotoxic activity followed by cows and then the healthy humans. This step could be a start for identifying compounds responsible for cytotoxic activity in hope to explore new medicine with therapeutic activity against cancer.

Introduction

Natural substances like plant extracts, microbes-derived and marine organisms-derived agents, human and animals waste products are considered as the main resources for discovering of chemotherapeutic substances [1]. Animal feces consists of water, polysaccharides, proteins, undigested fats, microbial biomass, undigested food residues and ash The main elements in feces as a wet weight percentage includes oxygen (74%), hydrogen (10%), carbon (5%), and nitrogen (0.7%). Several previous reports have been studied the biological, chemical and physical nature of feces in healthy and diseased states and the variations among individuals using the parameters of age, gender and diet. The significant component of fecal mass consists nearly 10^{11} bacteria per gram in which most of them are still nonculturable; in addition to *Archaea* and fungi. Normal fecal pH is nearly neutral; it ranged 5.3–7.5 [2- 6]. The biological processes in feces have been detailed, that include both aerobic and anaerobic digestion of fecal contents and production of biogas [7, 8]. Animal feces was used in medicine as fecal transplantation for curing specific diseases [9].

In general, the cytotoxic activity viewpoint of animal feces has been evaluated slightly; early studies included the evaluation of the cytotoxic activity of fecal filtrates as a diagnostic tool for detection of cytotoxins of *Clostridium difficile*, *Campylobacter jejuni* or *Campylobacter coli* and other enteric pathogens associated with diarrhea in adults and children [10-12]. It was shown that diets have greatly influenced the cytotoxic activity of fecal filtrates such as high fat diets in relation with bile acid secretions [13, 14]. The

dietary components, the presence of digestible and undigestible food constituents, and microbial compositions have been mentioned to be related with cytotoxicity levels of the fecal water in animal's gut [15, 16]. Also, microbiota and the host-microbiota interactions in animal feces were assumed as important resources for identifying new bioactive products and the discovery of a lot of cytotoxic compounds that went to earn clinical importance [6, 17-19].

Nowadays, the majority of the familiar commercial antibiotics, industrial enzymes and organic acids in addition to antitumor agents are produced by microbes; the genetic profile of microorganisms can encode varieties of bioactive metabolites leads to the discovery of numerous compounds with therapeutic activity [20]. There are little attempts to explore medicinal therapeutic products from wastes materials and animal feces, except the digestion of mixed wastes into biogas, the existent procedures usually used single microbial inoculum, single species, but it is quite difficult to use mixed cultures. Theoretically, mixed culture facilitates natural bioconversion regarding that the natural habitats contain unculturable bacteria in addition to culturable ones, this may be with unlimited values in the field of biotechnology [21].

Cancer is a large group of diseases that occurred due to uncontrolled cell growth inside the body [22]. The GLOBOCAN 2018 guesses indicated that there were 18.1 million new cases of cancer and 9.6 million deaths all over the world in 2018, 1/8 men and 1/10 women were develop the disease during life span [23]. Therefore, there is desperate need to explore and develop alternative anticancer agents from natural sources [24] such as mixed-cultures of feces as natural bioreactors for the treatment of this dangerous disease.

Natural products have played an important role in treating and preventing human diseases, natural products with medicinal value have come from various sources such as plants, animals and terrestrial and marine microorganisms [25]. Genomic studies indicate that certain groups of bacteria and fungi have dozens of secondary metabolite pathways that are not expressed under standard laboratory growth conditions but under optimizing laboratory or natural conditions with mixed fermentation where the presence of neighboring microbes may induce secondary metabolite synthesis which increased the yields of previously detected and still undetected metabolites in addition to the induction of unexpressed pathways for bioactive constituents [26].

The mixed-cultures of bacteria, fungi and bacteria with fungi have led to the production of tremendous active molecules, increasing the production of constitutively present compounds and the accumulation of natural products with cytotoxic activity [27]. The host-microbiota interactions produce compounds that might act as stimulating keys and signals that trigger the production of some induced bioactive compounds by their microbial community [28]. Animal feces contain mixed microbial population, so that it may considered as natural bioreactors with natural medium and culturable and unculturable microbes. The microbial activity in animals' gut may also contribute the response to certain diseases include those related with imbalance microbiota [29, 30]. Regarding the cytotoxic activity; microbial metabolites have direct anti-cancer effects such as anthracyclines, doxorubicin and actinomycin D [31].

Feces represents a medium for a natural mixed-microbial culture in addition to that it by itself represents a bioproduct. Thus, the aim of this current study was to utilization of human and animal feces as natural bioreactors to explore the cytotoxic activity of the cell-free fecal filtrates of healthy human subjects and colorectal cancer patients along with feces of healthy dogs and cows in hope to open a door to search for the therapeutic value of fecal components against serious diseases like cancer.

Materials and Methods

Materials

Human subjects

Fifteen healthy individuals including 7 males and 8 females, aged between 20 to 40 years, who were not administrated with antibiotics for at least 1 year before being involved in this study. Ten colorectal cancer patients including 5 male and 5 females, aged between 24 to 70 years whose cancer stages were 3 and 4 were also included in this study.

Dogs

Ten healthy Russian Alaska male dogs at Serchinar Zoo, Sulaimaniyah city were used for this purpose.

Cows

Ten healthy local bred female cows of a village flock at rural district of Sulaimaniyah Province were enrolled in this study.

Sample collection

Human, dog, and cow fecal samples were collected within the period 11th November to 1st December 2018. Persons were instructed to collect their feces using wood sticks and sterile plastic containers. Dog and cow fresh feces were collected using disposable plastic tea spoon and sterile plastic containers. The pH of the specimens ranged (6.5-7.1) for dog, (5.9-6.6) cows, (5.1-7) healthy humans, and (6.9-7.3) for colorectal cancer patients. Samples were transferred immediately to laboratory to prepare fecal cell- free.

Cell line

HeLa human cervical cancer cells were purchased as T25 flask by Fine Biotech (ATCC, China) that maintained under aseptic conditions. The cells were seeded in complete growth medium composed of 89% high glucose DMEM, 10% fetal bovine serum (FBS) and 1% antibiotic (a mixture of pencillin+streptomycin). All these supplements were purchased fromTransGen Biotech, China. The cells were incubated under humidified atmosphere at 37 °C and 5% CO2 for propagation and confluences.

Preparation of fecal cell- free filtrate

The procedure of Cover et al. (1990) [11] was adopted properly. Briefly, the stool specimen was weighted, mixed thoroughly with phosphate buffer saline (PBS) (pH 7.4) at a ratio of 1:3 (w/v) in a plastic sterile container and homogenized. Then, each fecal suspension was centrifuged 3 times at 6000 rpm for 10 minutes. Later on, the supernatant was passed 3 times through a Whatman No.1 filter paper. Additionally, the filtrate was passed twice through the sterile syringe membrane filter 0.45 and then 0.22 µm to obtain cell-free filtrate. All steps were achieved aseptically then the sterility of cell- free fecal filtrate was checked for the presence of living bacteria and fungi by culturing of 20 µL on nutrient agar and potato dextrose agar. Finally, cell-free filtrates were preserved at -25° C.

Cell viability determination using MTT assay

Viable cells were measured using MTT assay that depends on the cleavage of the tetrazolium salt that metabolized by the active cells, the MTT 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) is yellow water soluble formazan reduced to purple formazan. The quantity of MTT-formazan can be estimated by spectrophotometer [32, 33]. MTT solution was prepared by dissolving it in PBS at a concentration of 5 mg/ml and sterilized by 0.22 µm Millipore syringe filter. Before seeding the cells, % cell viability was checked by Trypan blue assay then 200 µL cell-medium suspension seeded onto 96 wells cell culture plate (20.000 cells per well) and incubated for 24 hours in humidified atmosphere at 37 °C and 5% CO2. The morphology of the cells was observed under an inverted microscope before treated with the fecal filtrates. After 24 hours of incubation, the old medium was discarded though automatic aspirator, cells were fed with 100 µL of fresh medium and 100 µL from different concentrations of 50 mg/ml fecal filtrate stock solution (100, 200, 400, 800, 1600, 3200, 6400 µg/ml), then the treated wells were incubated for 24 hours at 37° C and 5% CO2. The morphology also observed under inverted microscope after exposure to different fecal concentrations. After incubation, the old medium and filtrate in each well were removed out, then the cells were fed with 200 µL fresh medium and 50 µL MTT solutions (5 mg/ml in PBS). The plates were incubated for additional 4 hours in dark, after incubation the medium and the MTT were removed out and replaced by 200 µL of dimethyl sulfoxide (DMSO). The plates were incubated in shaker incubator for 15 minutes. The absorbance was read at 600 nm using ELISA plate reader. Positive control was the treating wells with concentrations of 5-fluorourasil (40, 80, 160, 320 and 400 µg/ml). Normal growth control wells contain cell-medium suspension while blank wells contain only medium. The experiment performed in 3 replicates and repeated 3 times. The growth viability (%) of cells was calculated according to the following equation:

Cell viability (%) =Absorbance of treated well / absorbance of untreated well × 100.

Size exclusion chromatography

Crude cell-free fecal filtrates were passed through 0.22 μm Millipore syringe filter. Sephadex G 100 cross linked dextran was soaked in the buffer (0.05 M sodium phosphate and adjusted by NaCl until pH 7.4) and let to swell for 48 hours, unswelled beads and fine particles were removed by decantation then the swelled gel was poured into long and thin column (50 cm in length and 1 cm in diameter). Then each fecal filtrate was loaded in circular motion around the inside of the column. Various fractions of about 3 ml were collected with a flow rate adjusted at 0.5 ml (10 drops) per minute then the OD of each fraction was monitored at 280 nm using UV-visible spectrophotometer (Cecil Aquarius, UK) with 10 mm path length cuvette. A background spectrum for buffer solution was obtained and subtracted from spectra of sample fractions [34, 35].

Cell viability of fractions using MTT assay

The colorimetric assay mentioned above was also used for fractions of each fecal filtrate in which different volumes (100, 50, 25, 12.5 μL) from each fraction were added into wells that contained 20.000 cells. Finally, the absorbance was measured at 600 nm, the growth viability (%) was calculated as below:
Cell viability (%) = Absorbance of treated well / absorbance of untreated well × 100.

Statistical analysis

Using two-way ANOVA as a general test for percentage viability of 4 fecal samples each with 7 concentrations using complete randomized design (CRD) and means of the factors were compared according to least significant deference (LSD 0.01).

Results

Cytotoxic activity of crude cell-free fecal filtrates

The cytotoxic activity of the crude specimens of each fecal filtrate for the 4 different fecal samples against HeLa cells using MTT colorimetric assay revealed that the IC50 is dose- dependent which means the cell killing effect is corresponded to increase according to fecal filtrate concentration. IC50 of healthy human, colorectal cancer, dog and cow crude fecal filtrates and 5-FU were 1715 ± 56.9, 400.76 ± 32, 442.64 ± 23.29, 1265 ± 35.8, and 134.33 ± 3.29 respectively (Figure 1 a, b, c and d). Moreover, the statistical analysis showed that the differences among the different samples in term of percentage viability of HeLa cells were highly significant ($P<0.001$). The differences among concentrations of each fecal cell-free filtrate of the 4 samples were highly significant with respect to percentage viability of HeLa cells ($P<0.001$), whilst there were no significant differences in interactions among samples and their concentrations ($P> 0.05$). It was obvious that colorectal and dog fecal filtrates were with higher cytotoxic effect than cow and healthy human samples where the mean % viability of all concentrations for colorectal cancer, dog, cow and healthy human fecal filtrates were 40.721%, 44.637 %, 55.205% and 60.958%, respectively (Table 1).

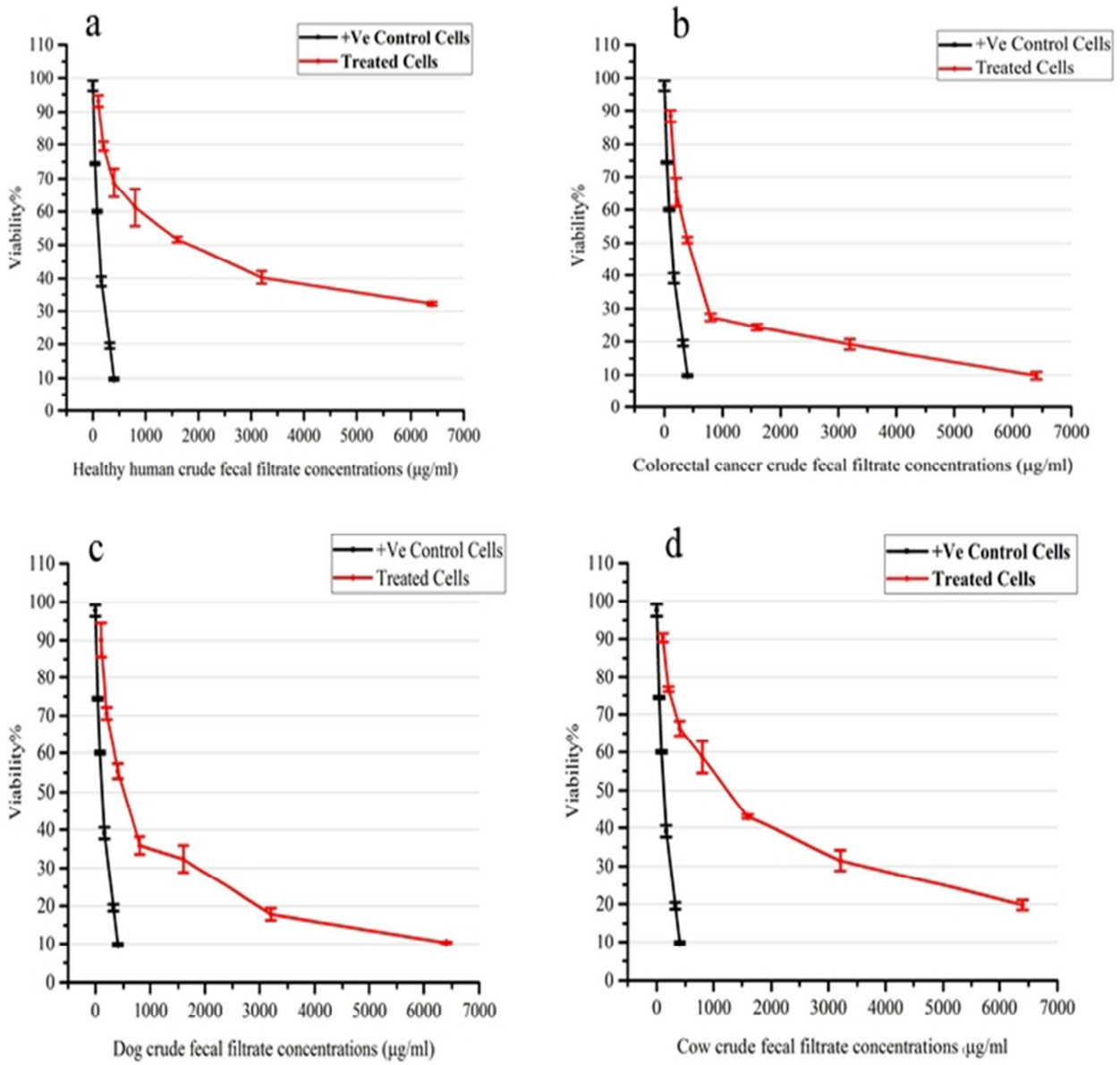

Figure-1: Effect of different concentrations of (a): healthy humans, (b): colorectal cancers, (c): dogs and (d): cows' crude cell-free fecal filtrate on %viability of HeLa cells. IC50 is 1715 ± 56.9, 400.76 ± 32, 442.64 ± 23.29 and 1265 ± 35.8 µg/ml respectively, assayed by colorimetric MTT assay. The positive (+ve) control is 5-fluorouracl.

Table-1: % viability of HeLa cancer cells treated with different sources of crude fecal filtrates, concentrations, and their interaction.

Source of fecal filtrates	***Concentrations***							***Mean samples % viability of HeLa cells***
	100	*200*	*400*	*800*	*1600*	*3200*	*6400 µg/ml*	
Dogs	*90.022*	*70.598*	*55.340*	*35.906*	*32.416*	*17.886*	*10.292*	*44.637 %*
Cows	*90.328*	*76.734*	*66.352*	*58.677*	*43.005*	*31.525*	*19.811*	*55.205 %*
Healthy humans	*88.295*	*65.281*	*50.967*	*27.314*	*24.247*	*19.163*	*9.780*	*60.958 %*
Colorectal patients	*88.295*	*65.281*	*50.967*	*27.314*	*24.247*	*19.163*	*9.780*	*40.721 %*
*Mean of samples concentrations on% viability of HeLa cells (***P < 0.001)*	*90.443*	*73.030*	*60.324*	*45.796*	*37.834*	*27.224*	*18.009*	*(***P < 0.001)*

Samples & Concentrations interaction (P > 0.05).

Size exclusion chromatography

Three ml of each of 11 fractions of dog fecal filtrate, 10 of cow, 11 of healthy human, and 12 of colorectal patients were collected. The significant peaks observed for each fecal sample (Figure 2).

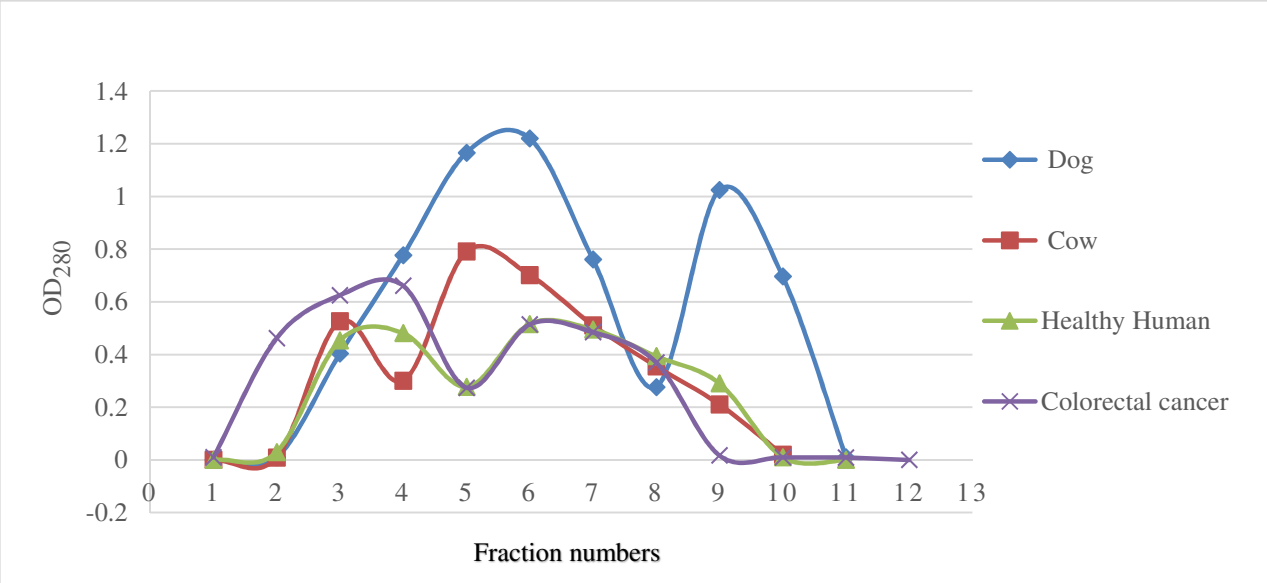

Figure-2: crude cell-free Fecal filtrates fractionation by size exclusion chromatography using sephadex G100 cross-linked dextran. Fractions were checked through UV-Visible spectrophotometer at 280 nm with 10 mm pathlength, buffer composition; 0.05 M sodium phosphate adjusted by NaCl (pH 7.4). Significant peaks observed for each filtrate.

Cytotoxic activity of fecal filtrate fractions

HeLa cells were exposed to different volumes of each fraction, 100, 50, 25 and 12.5 µL. Out of the 11 dog fecal filtrate fractions 4 fractions were cytotoxic active; the fractions F4, F5, F6 and F7 gave IC_{50} at 100, 13, 12 and 52 µL respectively (Figure 3), and these fractions located in the first peak of column chromatography. Out of 10 cow fecal filtrate fractions 3 were cytotoxic active; Fractions F3, F5 and F6 gave IC_{50} at 27, 25 and

73 µL respectively (Figure 4); F3 located in the first peak while F5 and F6 located in the second peak. Out of 11 healthy human fecal filtrate fractions 2 were cytotoxic active; fractions F3 and F4 gave IC_{50} at 13 and 12.5 µL respectively (Figure 5); both fractions are within the first peak. Out of 12 colorectal cancer fecal filtrate fractions 4 were cytotoxic active; fractions F2, F3, F4 and F6 gave IC_{50} at 26, 25, 16 and 24 µL, respectively (Figure 6), Cytotoxic activity was found in both peaks.

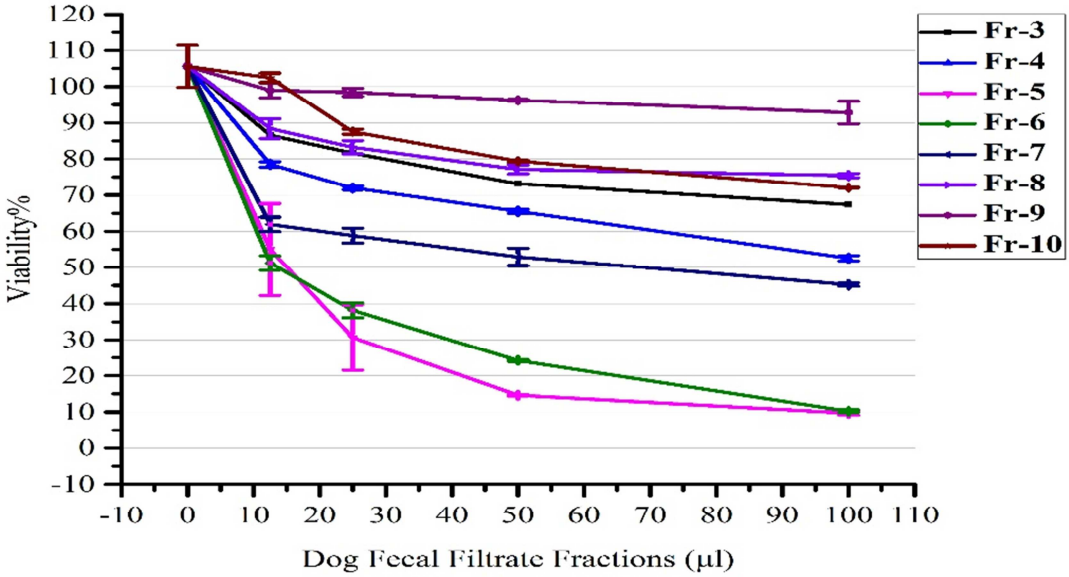

Figure-3: % viability of HeLa cells after treating with different volumes (100, 50, 25, 12.5 µL) of each of 8 dog fecal filtrate fractions of which the fractions F4, F5, F6 and F 7 were with IC_{50}.

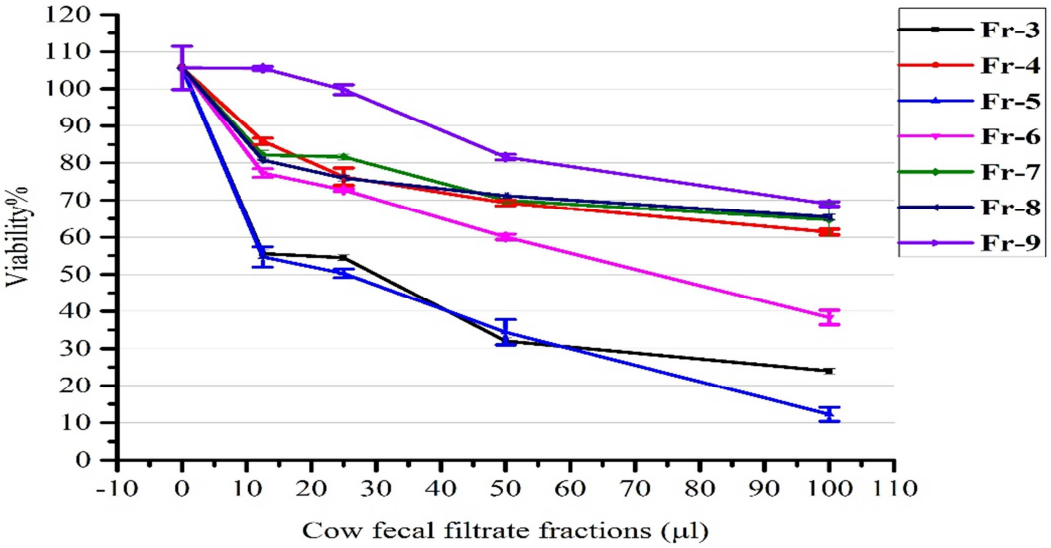

Figure-4: % viability of HeLa cells after treating with different volumes (100, 50, 25, 12.5 µL) of each of 7 cow fecal filtrate fractions of which the fractions F3, F5 and F 6 were with IC_{50}.

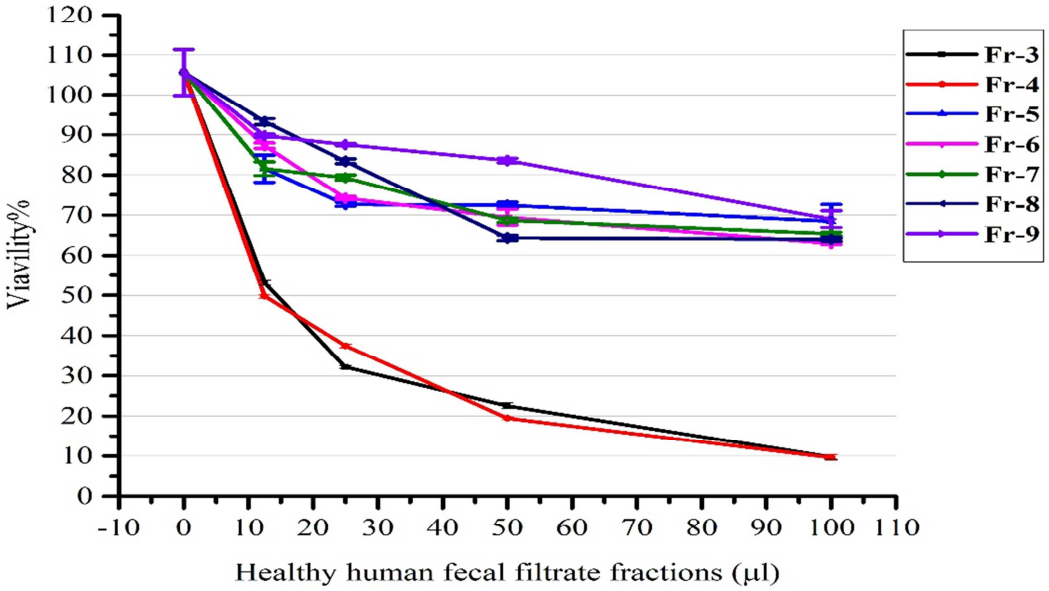

Figure-5: % viability of HeLa cells after treating with different volumes (100, 50, 25, 12.5 µL) of each of 7 healthy human fecal filtrate fractions of which the fractions F3 and F4 were with IC_{50}.

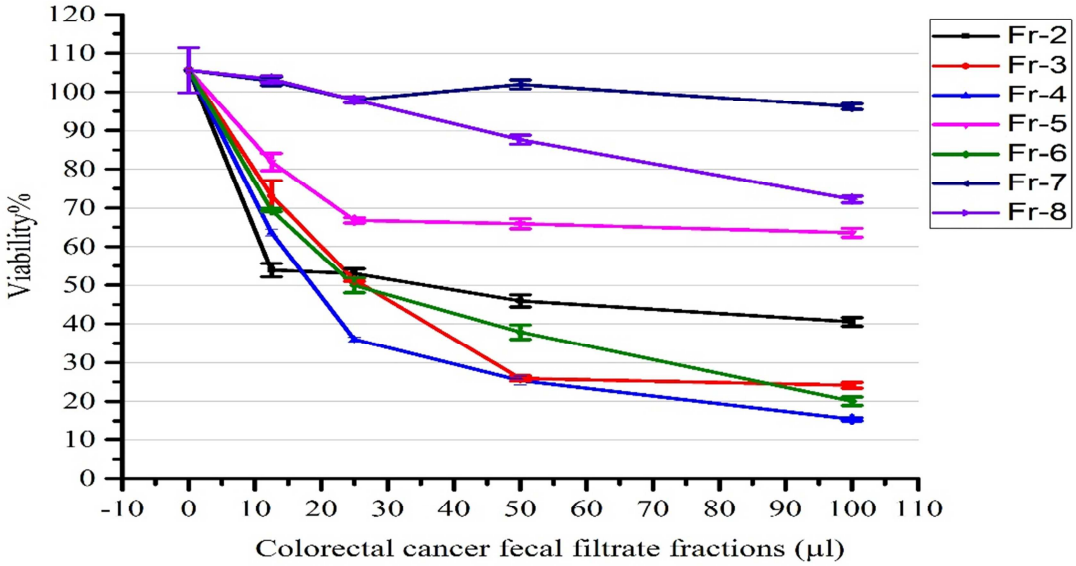

Figure-6: % viability of HeLa cells after treating with different volumes (100, 50, 25, 12.5 µL) of each of 7 colorectal cancer fecal filtrate fractions of which fractions F2, F3, F4 and F6 were with IC_{50}.

Discussion

Natural products as a part of medicinal treatments have been discovered in thousands of animals, plant, and microbial species [36]. Animal feces contains diverse microbial populations and their metabolic byproducts in addition to host-microbiota interactions that could make it a natural mixed culture for production of various products including anticancer agents [18, 19, 37]. Both crudes and fractions showed that colorectal and dog fecal filtrates are with higher cytotoxic activity followed by cow and healthy human fecal filtrates. These different cytotoxic activities among different fecal samples have been prospected in literatures for crude fecal filtrates to be due to the heterogenous and diverse microbial composition, types of

metabolic end products of both host and microbiota, physiochemical conditions across the gastro-intestinal tract (GIT), and the complex host-microbe interaction [4, 19, 38].

In fact, this study is an attempt to work on fractions hopping to identify the precise effective substances in future works. Cytotoxic activity among healthy dog, cow and human samples were significantly different. Both crudes and fractions indicated that dog which is a carnivorous and colorectal patients fecal filtrates have higher cytotoxic activity followed by the herbivorous cow and the omnivorous healthy human. So that, food consumed may have an effect with this respect. The IC_{50} of the fecal filtrate fractions revealed that 4 active fractions of dog, 3 of cow, 2 of healthy human and 4 of colorectal cancer patients may represent activity of different substances; According to UV-visible spectrophotometer peaks, dog active fractions appeared to be of large molecules in size, while that of cow and colorectal active fractions represent intermediate-sized and that of healthy human active fractions contain small-sized. Compositions of fecal filtrates regarding the presence of various sized molecules were agreed with the previous investigations; a study showed the differences of the active substances may due to different metabolic pathways at the GIT of the three different feed-dependent animals [39]. Microbial compositions of animals are different, also the commensal and mutual host-microbe relationship lead to produce a variety of molecules with different chemical structures and molecular weights [15]. Nützmann *et al*. (2011) [40] reported that mixed-microbial culture is a crucial approach in the possibility of producing diversity of bioactive compounds that have different cytotoxic effects. Marmann *et al.* (2014) [27] reported that mixed-culture of bacteria, fungi and bacteria with fungi lead to the production of tremendous active molecules and production of constitutively present compounds with remarkable cytotoxic activity on diverse cell lines that was not detected in monoclonal cultures. We assumed that diversification of natural products may be more accessible in mixed-culture; a previous study has showed that mixed fermentation have been used in production of bioactive compounds and industrial enzymes which led to increase the efficiency and the diversity of the products [41]. Some previously produced metabolites have been produced again in more amounts by using mixed fermentations, this was in addition to increase the yields of undiscovered metabolites, analogues of known metabolites resulting from combined pathways, and induction of previously unexpressed metabolic pathways for bioactive components [26]. Degenkolb *et al*. (2002) [42] suggested that Co-cultivation of microbes could produce different and structurally related compounds, so that this could be a possible way to diversify the microbial secondary metabolites by mixtures of different animals' microbial flora.

Natural and intact microbial habitat may induce microbial mixed-culture products; a previous study suggested that optimal environmental factors are essential to the biosynthesis of secondary metabolites [43] that means microbes in natural habitats metabolize more efficiently. In this study, the production of the colorectal cancer patients gut microbiota is indeed of non-intact habitat and so the production of the microbial action is supposed to be not optimal but it may be directed in response to the disease that may lead to produce compounds with notable cytotoxic activity. The results revealed that the colorectal fecal filtrate gave IC_{50} with a lower concentration in comparison to that of intact healthy human fecal filtrate and even the filtrates of dog and cow feces. In non-intact colorectal habitat the relationship among the microbiota may be troubled because of the effects of the alteration in physiological condition [44], poor feeding and/or treatment [45], that lead to increase the competitions among the normal microbiota. Previous studies revealed that the competition among different microbes due to limiting nutrient availability and other environmental stress is returned to the selective force through the induction of genes which involve in the synthesis of bioactive compounds [27, 47].

Pettit (2009) [26] suggested that in mixed culture some microbes required signals and growth factors from other microbes in order to turn on their genes that involve in biosynthesis of active compounds. Our opinions were the exploration for new bioactive compounds in feces as a natural bioreactor rather than to explore them by monoclonal cultures. Many biosynthetic fungal silent gene clusters that encode bioactive metabolites are highly induced when co-cultivated with bacteria, these interactions were happened in different environment such as soil, marine, food and even in the patient's body that lead to production of new compounds as well as their analogs and derivatives with observable antitumor activity such as libertellenones

and xanthocillin [46, 47, 48]. Besides that, mixed culture of fungi themselves could produce new metabolites; a study revealed the isolation of lateritin as a major metabolite byproduct from mixed fermentation of five sediment-derived fungi, lateritin has showed cytotoxic activity against many human tumor cell lines [49]. These findings support the prediction of this study about the exploration of expected novel bioactive compounds in feces regarding that a few studies have been conducted with this respect.

Kennedy *et al*. (1991) [50] have shown that fecal extracts usually have cytotoxic effects in tissue culture, they return the effects to the enteric pathogenic bacteria especially toxin-producing ones where they worked on children feces of diarrheal cases. the cytotoxic activity of the different fecal filtrate samples in the current study may be a result of bioactive compounds produced by the mixture of normal microbiota and host-microbial interactions rather than the effects of pathogenic bacteria since all animals subjected to sampling in this study were healthy even that the colorectal cancer patients were with no microbial infections. In fact, the composition of the microbiota and their impacts in dogs, cows, healthy humans and colorectal cancer patients' feces are significantly different as it was reported in the literatures to be due to the differences in diet which drives the diversity of microbiota and thus influence their functions [51]. Mixed bacterial flora in different animals may have a main role in cytotoxic activity of fecal filtrates; a previous study reported that the mixed bacterial cultures induce the synthesis of unidentified secondary metabolites such as Gram-Positive bacteria that considered as an extensive resource of novel compounds include abundant therapeutic molecules with anti-tumor, anti-cancer, and antibiotic activities [52]. Previous studies showed that the bacterial groups that dominate dog feces are lactobacilli, actinobacteria, fusobacteria, bacteriodes and bifidobacteria while Cow fecal flora is dominated by strict anaerobes like *Bacteroides* spp., *Clostridium* spp. and *Bifidobacterium* spp. as well as facultative anaerobes such as Actinobacteria and *Enterobacteriaceae* [53, 4, 54]. Healthy human microbiota is dominated by Bacteroidetes, enterococci and members of phylum Proteobacteria [51]. Fecal flora that dominate the colorectal cancer GTI are Actinobacteria, Firmicutes (Lactobacillales), Bifidobacterium, Fusobacterium and Enterobacteria [55, 56]. The dominated Dog and Cow feces microbiota have diverse biological activities as the members of Actinobacteria and lactobacilli that found nearly in all animals' body and feces, accounting to nearly more than 40% of all secondary metabolites of microbial origin which isolated from different natural sources, among the novel compounds that have anticancer activity are doxorubicin, actinomycin D, bleomycin, aclarubicin, mitomycin pentostatin and mitomycins [57, 58, 59]. On the other hand, on the assumption that the fecal filtrate may contain viruses, and according on the findings that have suggested most viruses have very tough requirements for their hosts in order to be cytotoxic which lead to no adversely effects on cell cultures of sources other than their hosts [60], the current findings mention to that the cytotoxic effect is not related to viruses, this is in addition to the fact that HeLa cell lines could express several atypical receptors which promote tumor proliferation and serve in immunological escape mechanism [61], so that alteration in cellular receptors in HeLa cells may challenge the attachment of viruses in spite of that it was mentioned previously to that "changes" in cell culture in response to fecal extracts are because of the presence of viruses [50].

The chemotherapy frequently alters the gut microbiota of colorectal cancer patients [44]. Those patients who were subjected to sampling at this study were administrated with two chemotherapies oxaliplatin and capecitabine, about 95.5% of the administered dose of Capecitabine is eliminated mainly through kidneys while fecal excretion represents only 2.6%. Oxaliplatin was found at 2% in patient's feces [62, 63], this indicate that the cytotoxic activity of the colorectal fecal filtrate is far from the impact of these two chemotherapies but may be related to alteration in physiology and poor feeding that lead to the induction of active compounds production as it was mentioned before. Assuming that the host-microbe relationship may produce bioactive substance, a study showed that commensal colon microbiota stimulates the immune system to produce bioactive peptides especially in pathological states through multistep biochemical processes which have various biological activities against foreign bodies inside the host [64, 65]. In addition to that the animal hosts produce compounds that might act as stimulating keys and signals trigger the production of some induced bioactive compounds by their microbial community [28].

Many biosynthetic gene clusters (BGCs) of microbiota that encode small molecules were identified in many microbial samples obtained from five body sites of healthy individuals, among them three classes which includes lantibiotics, thiopeptides, and thiozole were encodes by BGCs, these metabolites have cytotoxic activity on cancer cells [18, 19]. Garcia-Gutierrez et al. (2019) [66] explained that humans fecal normal floras could produce Non-ribosomal peptides (NRPs) and Ribosomally synthesized peptides that have diverse functions such as cytotoxic, antimicrobial and immunomodulatory effect. Bacteriocins are also known to have anticancer activity against various human cell lines such as breast cancer, colon cancer, bone cancer and HeLa cell line [67]. Jiang *et al*., (2013) [68] isolated 238 fecal actinobacterial strains from 31 wild animals and their fermentation extracts were showed broad-spectrum of cytotoxic activity against HepG-2, K562, HL60, Skov-3 and A431 cell lines. Ding *et al*. (2016) [69] identified and purified the new bioactive compounds (bafilomycins and odoriferous sesquiterpenoids) that isolated from healthy adult *Elephas maximus* fecal matter, the compounds gave potent anticancer activity various cell lines like human hepatocellular carcinoma (SMMC-7721 and colon adenocarcinoma (Caco-2). Whole cell extract of *Bifidobacterium adolescentis* isolated from feces of 20 healthy volunteers has been shown dose-dependent inhibitory activity against three Human colon cancer cell lines (Caco-2, HT-29, and SW480) [70]. Fermentation and Biotransformation of food components by mixed microbial flora are more likely to be happen in animal feces; previous studies showed that *Fusobacterium spp*., *Lactobacillus acidophilus, Bifidobacterium longum* and *B. lactis* could produce discrete new metabolites from the fermentation or biotransformation of nutritional constituents which that induce apoptotic activity in cancer cells, in rat and mouse animal model, the mentioned metabolites have been reported to prevent carcinogen-induced colon cancer proliferation and displayed growth elimination of transplantable melanomas with boosting of cytotoxic T cell -mediated immunosurveillance [15, 71, 72]. Lactic acid bacteria could prevent colon cancer through binding and degradation of potential carcinogens, antitumorigenic compounds production and metabolic activity alteration of other microbes inside GIT [73].

Conclusion

The bioactive compounds are of major interest due to their applicability as therapeutic activities. Microbial community has already played an essential role in human medicine especially the power of mixed-microbial fermentation for increasing the chemical diversity of microbial metabolites. It was concluded that colorectal patients and dogs' fecal filtrates have the higher cytotoxic activity followed by cows and healthy human against HeLa cell lines. The present study highlighted the importance of animals' fecal filtrates as natural bioreactors to produce cytotoxic compounds include antitumor activities that might be expected to play an important role in successful transition from observational and empirical screening towards separation, purification and new drugs discovery.

Acknowledgment

The authors would like to thank Kurdistan Institution for Strategic Studies and Scientific Research (KISSR). In Sulaimani city and to Iraqi Center for Cancer and Medical Genetics Research in Baghdad for technical assistance.

References

[1] Mann, J., "*Natural products in cancer chemotherapy: past, present and future*." Nature Reviews Cancer. Vol. 2, No. 2, pp.143-148. (2002).

[2] Rose, C., et al., *"The characterization of feces and urine: a review of the literature to inform advanced treatment technology*." Critical reviews in environmental science and technology. Vol. 45, No. 17, pp. 1827-1879. (2015).

[3] Heaton, K.W., et al., *"Defecation frequency and timing, and stool form in the general population: a prospective study"*. Gut. Vol. 33, No. 6, pp. 818-824. (1992).

[4] Simpson, J.M., et al., *"Characterization of fecal bacterial populations in canines: effects of age, breed and dietary fiber"*. Microbial ecology. Vol. 44, No. 2, pp. 186-197. (2002).

[5] Achour, L., et al., *"Faecal bacterial mass and energetic losses in healthy humans and patients with a short bowel syndrome"*. European journal of clinical nutrition. Vol. 61, No. 2, pp. 233. (2007).

[6] Leser, T.D. and L. Mølbak, *"Better living through microbial action: the benefits of the mammalian gastrointestinal microbiota on the host"*. Environmental microbiology. Vol. 11, No 9, pp. 2194-2206. (2009).

[7] Liang, C., K.C. Das, and R.W. McClendon, *"The influence of temperature and moisture contents regimes on the aerobic microbial activity of a biosolids composting blend"*. Bioresource technology. Vol. 86, No. 2, pp. 131-137. (2003).

[8] Guendouz, J., et al., *"High-solids anaerobic digestion: comparison of three pilot scales"*. Water Science and Technology. Vol. 58, No. 9, pp. 1757-1763. (2008).

[9] Aroniadis, O.C. and L.J. Brandt, "*Fecal microbiota transplantation: past, present and future*". Current opinion in gastroenterology. Vol. 29, No. 1, pp. 79-84. (2013).

[10] Ryan, R.W., I.R. Kwasnik, and R.C. Tilton, *"Rapid detection of Clostridium difficile toxin in human feces*". Journal of clinical microbiology. Vol.12, No. 6, pp. 776-779. (1980).

[11] Cover, T.L., G.I. Perez-Perez, and M.J Blaser, "*Evaluation of cytotoxic activity in fecal filtrates from patients with Campylobacter jejuni or Campylobacter coli enteritis*". FEMS microbiology letters. Vol. 70, No. 3, pp. 301-304. (1990).

[12] Ferreira, C.E.. V. Nakano and M.L. Avila-Campos, "Cytotoxicity and antimicrobial susceptibility of Clostridium difficile isolated from hospitalized children with acute diarrhea". Anaerobe. Vol.10, No. 3, pp. 171-177. (2004).

[13] Gullikson, G.W., W.S. Cline. V. Lorenzsonn, L. Benz, W.A. Olsen, and P. Bass, "Effects of anionic surfactants on hamster small intestinal membrane structure and function: relationship to surface activity". Gastroenterology. Vol. 73, No.3, pp. 501-511. (1977).

[14] Rafter, J.J., P. Child, A.M. Anderson, R. Alder. V. Eng, and W.R. Bruce, "Cellular toxicity of fecal water depends on diet". The American journal of clinical nutrition. Vol. 45, No. 3, pp. 559-563. (1987).

[15] Davis, C.D. and J.A. Milner, "*Gastrointestinal microflora, food components and colon cancer prevention*". The Journal of nutritional biochemistry. Vol. 20, No. 10, pp. 743-752. (2009).

[16] Mahmud, A., "*An investigation of the relationship between dietary fiber, fecal bacterial composition, and colon cancer.*" Ph.D. dissertation, Carleton University, Ottawa, Canada. (2012).

[17] Ley, R.E., et al., *"Evolution of mammals and their gut microbes"*. Science. Vol. 320, No. 5883, pp. 1647-1651. (2008).

[18] Arnison, P.G., et al., *"Ribosomally synthesized and post-translationally modified peptide natural products: overview and recommendations for a universal nomenclature"*. Natural product reports. Vol. 30, No. 1, pp. 108-160. (2013).

[19] Donia, M.S., et al., *"A systematic analysis of biosynthetic gene clusters in the human microbiome reveals a common family of antibiotics"*. Cell. Vol. 158, No. 6, pp. 1402-1414. (2014).

[20] Dan. V.M. and R. Sanawar, *"Anticancer agents from microbes"*. In Bioresources and Bioprocess in Biotechnology. pp. 171-184. (2017).

[21] Supaphol, S., et al., *"Microbial community dynamics in mesophilic anaerobic co-digestion of mixed waste"*. Bioresource technology. Vol. 102, No. 5, pp. 4021-4027. (2011).

[22] Gomathi, M. and P. Thangaraj, *"A computer aided diagnosis system for lung cancer detection using support vector machine"*. American Journal of Applied Sciences. Vol. 7, No. 12, pp. 1532. (2010).

[23] Bray, F., et al., *"Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries"*. CA: a cancer journal for clinicians. Vol. 68, No. 6, pp. 394-424. (2018).

[24] Rutledge, P.J. and G.L. Challis, *"Discovery of microbial natural products by activation of silent biosynthetic gene clusters"*. Nature reviews microbiology. Vol. 13, No. 8, pp. 509-523. (2015).

[25] Newman, D.J., G.M. Cragg, and K.M. Snader, *"The influence of natural products upon drug discovery"*. Natural product reports. Vol. 17, No. 3, pp. 215-234. (2000).

[26] Pettit, R.K., *"Mixed fermentation for natural product drug discovery"*. Applied microbiology and biotechnology. Vol. 83, No. 1, pp. 19-25. (2009).

[27] Marmann, A., et al., *"Co-cultivation—a powerful emerging tool for enhancing the chemical diversity of microorganisms"*. Marine drugs. Vol. 12, No. 2, pp. 1043-1065. (2014).

[28] Zhou, Y., et al., *"Alkaloids from the Sponge-Associated Fungus Aspergillus spp"*. European Journal of Organic Chemistry. Vol. 2013, No. 5, pp. 894-906. (2013).

[29] Louie, T. and P.C. Adams, *"Nature's therapy for recurrent Clostridium difficile diarrhea"*. Canadian Journal of Gastroenterology and Hepatology. Vol. 22, No. 5, pp. 455-456. (2008).

[30] Lozupone, C.A., et al., "*Diversity, stability and resilience of the human gut microbiota*". Nature. Vol. 489, No, 7415, pp. 220. (2012).

[31] Malla, S., et al., *"Limitations in doxorubicin production from Streptomyces peucetius"*. Microbiological research. Vol. 165, No. 5, pp. 427-435. (2010).

[32] Encalada, M.A., et al., *"Anti-proliferative effect of Melissa officinalis on human colon cancer cell line"*. Plant foods for human nutrition. Vol. 66, No. 4, pp. 328-334. (2011).

[33] Senthilraja, P. and K. Kathiresan, *"In vitro cytotoxicity MTT assay in Vero, HepG2 and MCF-7 cell lines study of Marine Yeast"*. Journal of Applied Pharmaceutical Science. Vol. 5, No. 3, pp. 80-84. (2015).

[34] Ó'Fágáin, C., P.M. Cummins, and B.F. O'Connor, *"Gel-filtration chromatography"*. In Protein Chromatography. Humana Press. pp. 25-33. (2011).

[35] Healthcare, G.E., *"Size exclusion chromatography: Principles and Methods"*. GE Healthcare. Handbooks, pp. 139. (2012).

[36] Da Rocha, A.B., R.M. Lopes, and G. Schwartsmann, *"Natural products in anticancer therapy"*. Current opinion in pharmacology. Vol. 1, No. 4, pp. 364-369. (2001).

[37] Frey, J.C., et al., *"Fecal bacterial diversity in a wild gorilla"*. Applied and. Environmental Microbiology. Vol. 72, No. 5, pp. 3788-3792. (2006).

[38] Donaldson, G.P., S.M. Lee, and S.K. Mazmanian, *"Gut biogeography of the bacterial microbiota*". Nature Reviews Microbiology. Vol. 14, No. 1, pp. 20. (2016).

[39] Rowland, I., et al., *"Gut microbiota functions: metabolism of nutrients and other food components"*. European journal of nutrition. Vol. 57, No. 1, pp. 1-24. (2018).

[40] Nützmann, H.W., et al., *"Bacteria-induced natural product formation in the fungus Aspergillus nidulans requires Saga/Ada-mediated histone acetylation*". Proceedings of the National Academy of Sciences. Vol. 108, No. 34, pp. 14282-14287. (2011).

[41] Salmon, I. and A.T. Bull, *"Mixed-culture fermentations in industrial microbiology"*. Current perspectives in microbial ecology, pp. 656–662. (1984).

[42] Degenkolb, T., et al., *"Formation of new lipoaminopeptides, acremostatins A, B, and C, by co-cultivation of Acremonium sp. Tbp-5 and Mycogone rosea DSM 12973"*. Bioscience, biotechnology, and biochemistry. Vol. 66, No. 4, pp. 883-886. (2002).

[43] Knight. V., et al., *"Diversifying microbial natural products for drug discovery"*. Applied Microbiology and Biotechnology. Vol. 62, No. 5-6, pp. 446-458. (2003).

[44] Zwielehner, J., et al., *"Changes in human fecal microbiota due to chemotherapy analyzed by TaqMan-PCR, 454 sequencing and PCR-DGGE fingerprinting"*. PloS one. Vol. 6, No. 12, p. e28654. (2011).

[45] Nourissat, A., et al., *"Relationship between nutritional status and quality of life in patients with cancer"*. European journal of cancer. Vol. 44, No. 9, pp. 1238-1242. (2008).

[46] Netzker, T., et al., *"Microbial communication leading to the activation of silent fungal secondary metabolite gene clusters"*. Frontiers in microbiology. Vol. 6, pp. 299. (2015).

[47] Oh, D.C., et al., *"Libertellenones A–D: Induction of cytotoxic diterpenoid biosynthesis by marine microbial competition"*. Bioorganic & medicinal chemistry. Vol. 13, No. 17, pp. 5267-5273. (2005).

[48] Zuck, K.M., S. Shipley, and D.J. Newman, *"Induced production of N-formyl alkaloids from Aspergillus fumigatus by co-culture with Streptomyces* peucetius". Journal of natural products. Vol. 74, No. 7, pp. 1653-1657. (2011).

[49] Pettit, R.K., et al., *"Isolation of human cancer cell growth inhibitory, antimicrobial lateritin from a mixed fungal culture"*. Planta medica. Vol. 76, No. 05, pp. 500-501. (2010).

[50] Kennedy, E., et al., *"Cytotoxic effects of children's faeces: relation to diarrhoea due to Clostridium difficile and other enteric pathogens"*. Annals of tropical paediatrics. Vol. 11, No. 2, pp. 107-112. (1991).

[51] Muegge, B.D., et al., *"Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans"*. Science. Vol. 332, No. 6032, pp. 970-974. (2011).

[52] Doroghazi, J.R., et al., *"A roadmap for natural product discovery based on large-scale genomics and metabolomics"*. Nature chemical biology. Vol. 10, No. 11, pp. 963. (2014).

[53] Clapper, W.E. and G.H. Meade., *"Normal flora of the nose, throat, and lower intestine of dogs"*. Journal of bacteriology. Vol. 85, No. 3, pp. 643-648. (1963).

[54] Dowd, S.E., et al., *"Evaluation of the bacterial diversity in the feces of cattle using 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) "*. BMC microbiology. Vol. 8, No. 1, pp. 125. (2008).

[55] Vargo, D., M. Moskovitz, and M.H. Floch, *"Faecal bacterial flora in cancer of the colon"*. Gut. Vol. 21, No. 8, pp. 701-705. (1980).

[56] Meng, C., et al., *"Human gut microbiota and gastrointestinal cancer"*. Genomics, proteomics & bioinformatics. Vol. 16, No. 1, pp. 33-49. (2018).

[57] Bérdy, J., *"Bioactive microbial metabolites"*. The Journal of antibiotics. Vol. 58, No. 1, pp. 1. (2005).

[58] Newman, D.J. and G.M. Cragg, *"Natural products as sources of new drugs over the last 25 years"*. Journal of natural products. Vol. 70, No. 3, pp. 461-477. (2007).

[59] Olano, C., C. Méndez, and J.A. Salas, *"Antitumor compounds from actinomycetes: from gene clusters to new derivatives by combinatorial biosynthesis"*. Natural product reports. Vol. 26, No. 5, pp. 628-660. (2009).

[60] Invitrogen, G., *"Cell culture basics"*. Life technologies, Gibco. pp. 16-18. (2014).

[61] Gutiérrez-Hoya, A., et al., *"Cervical Cancer Cells Express Markers Associated with Immunosurveillance"*. Journal of immunology research. Vol. 2019, pp. 10-20. (2019).

[62] Lévi, F., et al., *"Oxaliplatin"*. Clinical pharmacokinetics. Vol. 38, No. 1, pp. 1-21. (2000).

[63] Dhillon, S. and L.J. Scott, *"Capecitabine"*. Drugs. Vol. 67, No. 4, pp. 601-610. (2007).

[64] Gallo, R.L. and L.V. Hooper, *"Epithelial antimicrobial defense of the skin and intestine"*. Nature Reviews Immunology. Vol. 12, No. 7, pp. 503. (2012).

[65] Abt, M.C. and E.G. Pamer, *"Commensal bacteria mediated defenses against pathogens"*. Current opinion in immunology. Vol. 29, pp. 16-22. (2014).

[66] Garcia-Gutierrez, E., et al., *"Gut microbiota as a source of novel antimicrobials"*. Gut microbes. Vol. 10, No. 1, pp. 1-21. (2019).

[67] Kaur, S. and S. Kaur, *"Bacteriocins as potential anticancer agents"*. Frontiers in pharmacology. Vol. 6, pp. 272. (2015).

[68] Jiang, Y., et al., "Diversity and bioactivity of cultivable animal fecal actinobacteria". Advances in Microbiology. Vol. 3, No. 01, pp. 1. (2013).

[69] Ding, N., et al., *"Bafilomycins and odoriferous sesquiterpenoids from Streptomyces albolongus isolated from Elephas maximus feces"*. Journal of natural products. Vol. 79, No. 4, pp. 799-805. (2016).

[70] Lee, D.K., et al., *"Anti-proliferative effects of Bifidobacterium adolescentis SPM0212 extract on human colon cancer cell lines"*. BMC cancer. Vol. 8, No. 1, pp. 310. (2008).

[71] López de Felipe, F., B. de Las Rivas, and R. Muñoz, *"Bioactive compounds produced by gut microbial tannase: implications for colorectal cancer development"*. Frontiers in microbiology. Vol. 5, pp. 684. (2014).

[72] Zitvogel, L., et al., *"Anticancer effects of the microbiome and its products"*. Nature Reviews Microbiology. Vol. 15, No. 8, pp. 465. (2017).

[73] Hirayama, K. and J. Rafter, *"The role of probiotic bacteria in cancer prevention"*. Microbes and infection. Vol. 2, No. 6, pp. 681-686. (2000).