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Potential in Bioethanol Production from Various Agro Wastes Fermenting by Microorganisms using Carrot Peel, Onion Peel, Potato Peel and Sugar Beet Peel as Substrates

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Abstract

Large amount of agro wastes is produced in Rwanda each year. The global annual potential bioethanol production from the major vegetables wastes such as carrot peel, onion peel, potato peel and sugar beet peel were estimated. Those wastes processing was successfully used as raw materials for the production of bioethanol, employing by cellulase produced from various filamentous fungi including *Cladosporium cladosporioides* was used for hydrolysis and the fermentation of the hydrolyzed samples was done using *Saccharomyces cerevisiae*. The fermented product was purified by primary distillation process at 79°C and the fraction was collected. The ethanol is then determined by specific dichromate method and Gas Chromatography. Instantaneous saccharification and fermentation process yielded maximum ethanol in the substrate of carrot peel was 16.9% at 21st day and further confirmed by Gas chromatography and the yield of ethanol obtained was 15.8%.

1. Introduction

During oil crisis in 1970s a rising crude oil price, political instability and unstable oil market in countries producing oil and climatic changes, biomass has high potential to replace the supply of energy **(Nagashima *et al.,* 1984).** The amount of wastes produced by society each day is increasing in line with increasing populations worldwide and Rwanda is no exception. Agro wastes are an important part of total solid wastes produced nationally; they have potential to become an environmental pollution or more logically, to be utilized for the production of energy and other products.

Production of bioethanol from lignocelluloses materials such as agro wastes can substitute fossil oil production. Today, raw materials producing bioethanol by fermentation are classified as sugars, starches and cellulosic materials because fermentation is cheaper and easily than other fermentation (Bailey, 1986). The potential of bioethanol production from agro wastes of four crops which are carrot, onion, potato and sugar beet have been investigated. Currently, agro wastes are burnt by the rural farmers as cookers in households. Production of bioethanol from agro wastes have been attempted with enzymes from different sources for hydrolysis of biomasses and with different organisms for fermentation **(Öhgren *et al.,* 2006; Eken-Saracoglu and Arslan 2000).**

The demand for bioethanol is expected to increase dramatically until 2020 where there is an increase in the world population with expected 9 billion in the year 2050 increasing the need for food and energy **(Galal *et al.,* 2014)**. *S. cerevesiae,* also known as brewer's yeast, is the most commonly used fermentation microbe because of the baking and beer brewing industries **(Michalka, 2007; Roehr, 2001)**. Many of the sugar crops that would be suitable for industrial fermentation include sugar cane, sugar beet, fruits, sweet potato, sweet sorghum, Jerusalem artichokes and agro wastes **(Atiyeh and Duvnjak, 2002; Pramanik, 2005).**

The objective of this study was producing bioethanol from carrot peel, onion peel, potato peel and sugar beet peel for submerged fermentation and management system to maximize economic benefits at the same time protection of the environment.

2. Material and methods

2.1 Raw materials

Carrot peels, onion peels, potato peels and sugar beet peels, were collected from the local restaurant in volcanic region at early morning. They were clean to make free from sand, stone and dust by washing it twice in water. They were sun dried then each raw material was grinded and sieved into a 1mm. Those agro wastes are favorable for bioethanol production due to their availability and cheapest throughout the year.

2.2 Microorganisms producing ethanol

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Several bacteria, yeasts, and fungi have been used for bioethanol production. *S.cerevisiae*, is the most yeast, which can produce ethanol of the fermentation broth. The yeast *S. cerevisiae* can produce bioethanol up to 18% of the fermentation broth, **Pretorius (2000).**

Figure 1. Raw materials: agro wastes

2.3 Sources of microorganism

The isolated fungi were done from the rhizosphere of strawberry fields of College of Agriculture, Animal Science and Veterinary Medicine (Busogo) identified by serial dilution and wet mount technique **(Aneja, 2005).**

2.4 Culture medium chemical

The fermentation used was 0.2% yeast extract, 0.2% $(NH_4)\ NO_3$, 0.1% $MgSO_4{\cdot}7H_2O$, 0.2% KH_2PO_4 **(El- Gendy *et al.,* 2013)** and 5 g powdered of each substrate has been added.

2.5 Enzyme molecular weights

Poly-Acrylamide Gel Electrophoresis (PAGE) of the partial purified cellulase enzyme was performed according to **(Uk, 1970).** After electrophoresis, the gel was immersed in fixing solution. Staining of the band was done with coomassie brilliant blue, R-250 (CBB) for 2 h and later de-stained. The molecular weight of the cellulase was estimated using standard protein molecular weight marker consisting of Bovine Serum Albumin.

2.6 Protein estimation

The protein from partially purified samples of carrot peel, onion peel, potato peel and sugar beet peel were estimated **(Bradford,1976)** method. Optical density of the reaction mixture was observed at 660 nm against a blank prepared with 0.1 mL buffer.

2.7 Fermentation

Culture filtrate was further inoculated with *S. cerevisiae* and allowed for fermentation for 14^{th}, 21^{st} and 28^{th} days. After fermentation, it was filtered and ethanol content was determined **(Caputi, 1968).** As part of this study, we have reported a process for producing ethanol from agro wastes pre-hydrolysed by alkali followed by saccharification carried by co-cultivation of *C. cladosporioides* and fermentation of the released sugars to ethanol, using *S. cerevisiae* for ethanol production.

2.8 Distillation process

Distillation was carried in rotary vacuum flask at 80°C (boiling point of ethanol) and fraction is collected **(Kumnuanta *et al.,*1983)** as shown on Fig 2.

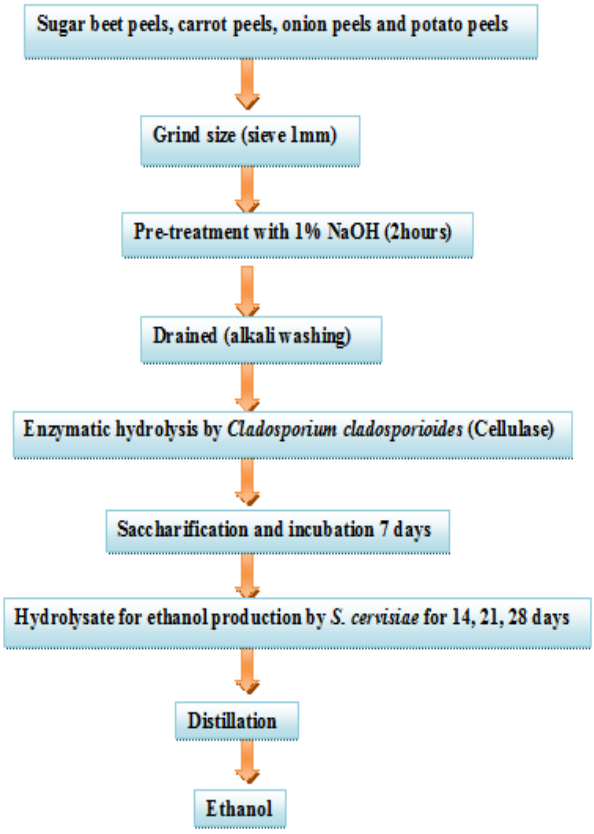

Figure 2. Bioethanol production from onion peel, sugar beet peel, carrot peel and potato peel.

2.9 Bioethanol estimation by potassium dichromate method

Standard ethanol was prepared from concentrations of 2% to 10% with blank. 2.5 ml of freshly prepared potassium dichromate solution (1g of potassium dichromate in 100 ml of pre-chilled $6H_2SO_4$) was mixed with 15ml of distillates and standards (2%, 4%, 6%, 8% and 10%) taken in separate test tubes and were incubated at 60ºC for 30 minutes (for color appearance) **Caputi, (1968).** Tubes were allowed to cool to room temperature and absorbance was estimated at 600nm **(William, 1950).**

2.10 Determination of quantity of ethanol produced

The distillate collected was measured using a measuring cylinder and expressed as quantity of ethanol produced in g/l by multiplying the volume of the distillate by the density of ethanol ($0.8033 g/cm^3$) **(Humphrey *et al.,* 2007).**

2.11 Confirmative analysis of ethanol by Gas Chromatography (GC)

The confirmation of ethanol qualitatively and quantitatively was done by gas chromatography method (Shimadzu Tokyo Japan). Gas Chromatography settings and characteristic features were selected to enable ethanol separation from the injected supernatant. 0.5 ml supernatant was dispensed into l ml capped sample and mixed with 5 ml of 1% internal standard solution. After mixing, 0.1 µL of the sample was directly injected into the Gas Chromatography **(Wang *et al.,* 2003).**

2.12 Statistical analysis

MS Excel version 2007 was employed for all statistical analysis. Data was recorded in triplicates and represented as a mean value.

3. Results and discussion

Currently bioethanol is produced from alcoholic fermentation of molasses or simple sugar, which are produced from crops generating starch or sugar. While technologies to produce ethanol from simple carbohydrates are well established, the technologies to produce bioethanol from agro wastes are still under development. It is possible that agro waste products may be economically converted to bioethanol. We used agro wastes peel as a source of lignocellulosic substrate for ethanol production (Figure1 and 2).

3.1 Enzyme molecular weights

The protein present in various agro wastes substrates showed several bands ranged from 30 to 130 kDa. The crude protein extract of carrot peel which contains maximum yield concentration of bioethanol confirmed its homogeneity and protein was resolved on 5% stacking and 12% running gel. The molecular weight of the protein bands was 30 kDa and 130 kDa for carrot peel (Figure 3).

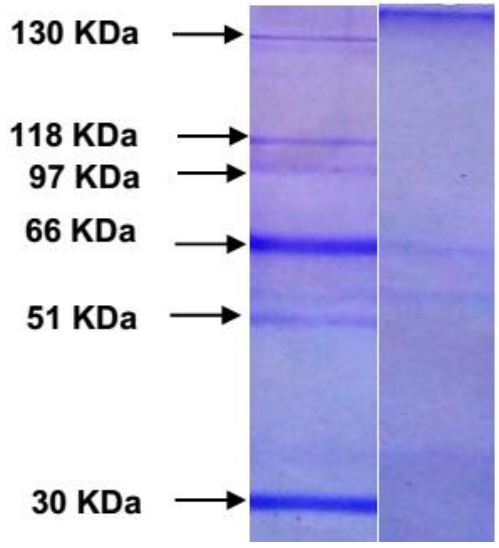

Figure 3. Molecular weight and cellulase activity from *Cladosporium cladosporioides*

Our results are close to the findings of **(Bai *et al.,*2013)** reported that the molecular weight of cellulase produced by different fungal species may vary from 12 kDa to 126 kDa. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is the most commonly used method for judging the apparent molecular weight of enzymes **(Ramani *et al.,* 2012).** Cellulase produced by *Trichoderma viride* was purified to homogeneity using DEAE-sepharose column and the molecular weight was estimated at 87 kDa by SDS-PAGE, **Yasmin *et al.,* (2013).** *Penicillium pinophilum* MS 20 produced a monomeric cellulase with molecular weight of 42 kDa, which appeared as a single band on SDS-PAGE gel **(Pol et al., 2012).** The cellulase produced by *Aspergillus niger* revealed a molecular weight of 60 kDa on SDS-PAGE gel **(Baraldo *et al.,* 2014).**

3.2 Protein estimation

The protein content with *C. cladosporioides* was observed in carrot peel 643.48 µg/ml, onion peel 1336.5µg/ml, potato peel 1318.76µg/ml and sugar beet peel 1101.12µg/ml (Table1). **Ado (2008)** reported the mycelial protein production by *Aspergillus niger* using banana peel. The protein content obtained by *Cladosporium sp* with lignocellulosic biomass was about 0.224 (mg/g) and mycelial protein of about 60.6±1.12 (mg/g) reported **(Mohan *et al.,* 2013).**

Table 1. Substrates protein content (µg/ml) with Cladosporium *cladosporioides*

Carrot peel protein content (µg/ml)	Onion peel protein content (µg/ml)	Potato peel protein content (µg/ml)	Sugar beet peel protein content (µg/ml)
643.48 µg/ml	1336.5 µg/ml	1318.76 µg/ml	1101.12 µg/ml

Mango peels ranged from 1.2258–13.8715 mg/ml in which *Aspergillus tamarii* produced the maximum protein concentration released on day 12 of cultivation. Watermelon peels, it ranged from1.8926–5.2474 mg/ml in which *Aspergillus terreus* gave the maximum biosynthesis potential on day 3 of fermentation. The yield of extracellular protein on the rampage on medium containing banana peels ranged from 0.9247-4.0108 mg/ml in which *Mucor piriformis* had the maximum biosynthesis potential on day 3 of submerged cultivation. Furthermore, on medium with plantain peels, it ranged from 1.1725-8.3441 mg/ml in which *Aspergillus sclerotio niger* had the maximum biosynthesis potential on day 3 of cultivation. Aspergillus *sp* take over *Fusarium sp* and *Mucor sp*. in polygalacturonase (PG) production.

3.3 Bioethanol obtained by dichromate method

Lignocellulosic materials and various agro wastes with different methods have been employed for bioethanol production. The maximum level of bioethanol varied from day-to-day fermentation. During the fermentation period, the ethanol yield of substrates was found to increase gradually from the 14th, 21st to 28th day (Figure 4). The maximum concentration of ethanol was achieved on 28th day of fermentation and started to level off. From the results obtained on bioethanol production potential of various lignocellulosic wastes varied and can be concluded that carrot peel was a very promising raw material for bioethanol production with *C. cladosporioides*. **Mishra *et al.,* (2012)** founded increase in quantity of ethanol produced in sub-merged state fermentation as compared to the produced by solid state fermentation and founded optimal incubation period 72 hours for bioethanol production by orange peel using *S. cerevisiae.* **Senthilkumar and Gunasekaran (2005)** reported that some gram-positive bacteria *Clostridium cellulolyticum, Lactobacillus casei* have been engineered for bioethanol production. **Dien *et al.,* (2003)** worked on Gram-negative bacteria Escherichia *coli, Klebsiella oxytoca,* and Zymomonas *mobilis. E. coli* and *K. oxytoca* are naturally able to use a wide spectrum of sugars, and work has concentrated on engineering these strains to produce ethanol selectively.

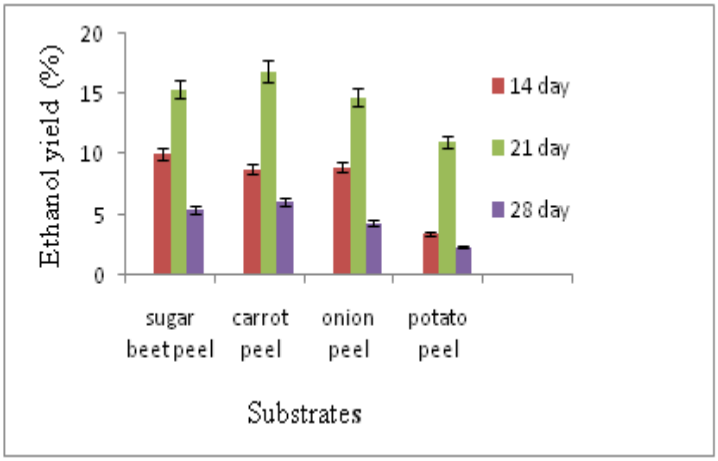

Figure 4. Ethanol obtained by dichromate method bioethanol yield (%) with agro wastes peel after 14th, 21stand 28th days of incubation of *Cladosporium cladosporioides*

3.4 Gas Chromatography and Bioethanol concentration

The Purity level for the ethanol through Gas Chromatography for the sample carrot peel was found to be to be 15.85%, the Retention Time [min] was 1.06, and Area [mV.s] was 2400, Height [mV] 168.364 and Area [%] 93.4 (Figure 5).

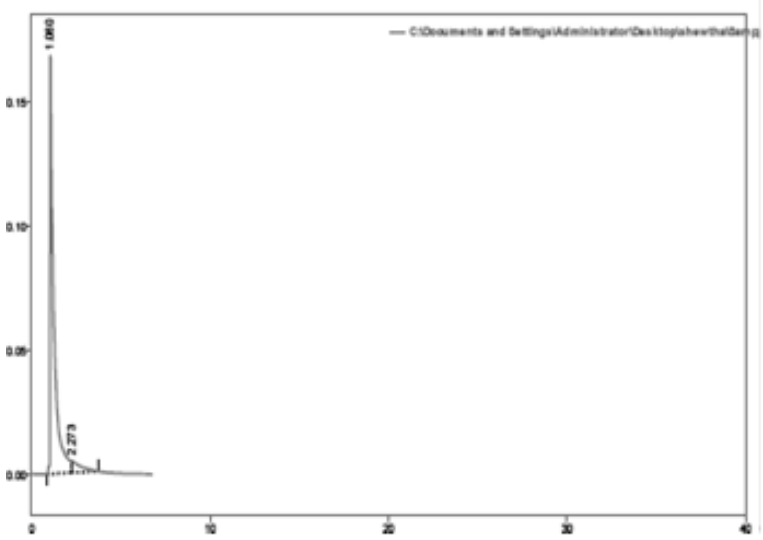

Figure 5. Gas Chromatogram of *Cladosporium cladosporioides* with carrot peel at 28 days

Isaie M and Padmavathi T (2016) reported that agro wastes such as carrot peel, onion peel, potato peel and sugar beet peel are products subjected to saccharification process by Penicillium *sp.* for the hydrolysis, this process was followed by the fermentation using yeast *S. cerevisiae* for the production of alcohol which was fermented at 14, 21, 28 days to produce alcohol. High yield of ethanol was obtained from sugar beet peel 14.52% on 28th day and further confirmed by Gas chromatography and the yield of ethanol obtained on 28th day was 17.3%. **Muchtaridi *et al.*, (2012)** determined alcohol contents of fermented black tape ketan based on different fermentation time with three different methods. Methods used are specific gravity, refractive index and GC-MS. Alcohol concentration obtained by using specific gravity method at 3, 10, 17, 24, and 31 days of fermentation, respectively, are 3.17% v / v; 3.02% v / v; 3.63% v / v; 3 , 12% v / v; and 4.47% v / v, using the method of refractive index is 3.90% v / v; 3.69% v / v; 4.31% v / v; 3.80% v / v and 5.04% v / v, and using GC-MS method was 4.30 % v/v; 4.23 % v/v; 5.01 % v/v; 4.75 % v/v; and 5.34 % v/v. The variation of fermentation time obviously did not influence the produce of alcohol contents statistically.

Table 2. Bioethanol concentration by *Cladosporium cladosporioides* (g/l)

Substrates + *Cladosporium cladosporioides*	**Bioethanol yield (g/l) at 14th day**	**Bioethanol yield (g/l) at 21st day**	**Bioethanol yield (g/l) at 28th day**
Sugar beet peel	79.136	121.506	42.606
Carrot peel	69.353	133.341	47.734
Onion peel	7.101	115.983	34.400
Potato peel	27.615	87.026	18.620

From the results obtained on bioethanol production potential of various lignocellulosic wastes varied and can be concluded that carrot peel was a very promising raw material for bioethanol production with *C. cladosporioides.* The maximum bioethanol concentration obtained in carrot peel at 21st day by *C. cladosporioides* was 133.341 g/l (Table 2). **Oyeleke *et al.*, (2009)** reported that the maximum volume of ethanol (27.10 g/l) produced from guinea corn husk and millet husk (18.24 g/l) at the 120th hours with *Zymomonas mobilis.* **Agulejika *et al.*, (2005)** reported maximum ethanol yield at 120th hour from fresh fruit (64.01 g/l) and waste fruits (21.14 g/l) using *Zymomonas mobilis*. **Micheal and Rosaline (2000)** reported that the highest ethanol yield from fresh fruit was due to higher presence of fructose and glucose in fresh fruits. **Ismail *et al.*, (2012)** has reported yields of bioethanol 0.475 g/g to 0.51 g/g of the Wheat Straw and corn cobs, and hulls acid hydrolysate respectively. Using green algae, **(Trivedi *et al.*, 2013; Ge *et al.*, 2011), (Wu *et al.*, 2014)** obtained an ethanol yield of 0.45 g/g from *U. fasciata,* 0.44 g/g from Laminaria *japonica* and of 0.47 g/g from hydrolysate Gracilaria *sp.*

4. Conclusion

The present study examined the influences of fermentation period on ethanol production ability of *S. cerevisiae* using the carrot peel, onion peel, potato peel and sugar beet peel as substrates. The results of this study indicate incubation time for fermentation using *S. cerevisiae* which may enhance ethanol yield and minimize the cost of production could be obtained from agro wastes as substrates. Bioethanol production by *S. cerevisiae* may be used as successful alternative of *S. cerevisiae* in bioethanol production.

Acknowledgments

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Declaration of interest

The authors report no conflicts of interest.

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Anticancer Activity Assay of Nano-Fractional Compounds that Purified from Soil Actinomycetes

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Abstract

Background and objectives: Cancer remains a global problem of health, and has been recorded as one of the causes of death after heart disease Natural products from plants, the environment and microorganisms are leveraged for the purpose of fighting cancer. Actinobacteria have been recognized as main sources of bioactive natural products as early as in the 1950s, for which about half of the secondary metabolites revealed, including enzymes, antibiotics, immunosuppressive, and anti-tumor agents

Materials and methods: The methods of this study included isolated and identification of bacteria from soil samples and identified by morphology characters and biochemical test. Subjected extract of actinomycetes to HPLC purification then collected purified fractions then analyzed by GC-mass. After the fractions were mixed with liposome nanoparticles which tested activity on HT29 colon cancer cell line.

Results: The results of identification of bacterial isolates showed the colonies growing on a SNA medium were morphologically identified where the colonies were well-growth and had a gray color, not producing dyes in the medium. The results of the biochemical tests indicated that isolates were amylase, catalase, and gelatinase producing isolates and non-lipase producing, non H2S production and consuming urea, while the carbon consumption test indicated the isolates' ability to consume starch, glucose and sucrose respectively. While the results of preparative HPLC revealed that 4 fractions were collected with desired amounts of each compound when using fraction collector in depend on mobile phase system in analytical HPLC with (50 % HPLC-grade acetonitrile) at 254 nm and cycling up was employed to increase the separation efficiency. The chemical composition of the HPLC fractions using GC-MS showed the identification of many components example (Hexadeconic acid, Octadecanoic acid, ethyl ester and Fumaric acid). The results *of In vitro* anti-tumor cytotoxicity showed that all four nano purified fractions were applied on HT 29 colon cancer cells and exhibited significantly differences compared with control treatments of inhibition cells number, these data were used to calculate the values of IC50 (the inhibitory value of half the number for all nanofractions. the application of concentration with inhibition value and solved the equation to IC50 value were gained, which were (151.4,16.4 , 16.6 and 43.8 µg/ml) to four nano fractions respectively.

Conclusion: This study showed that the use of HPLC to purify the bacterial extract and then combine the purified fractions with the nanoparticles liposome has inhibited cancer cells with high efficacy.

1. Introduction

To date, cancer remains a global problem of health, and has been recorded as one of the causes of death after heart disease**(Are *et al.*,2019; Chalbatani *et al.*, 2019)**.There are many causes of cancer, including unhealthy lifestyles such as eating junk food, alcohol, smoking and losing physical fitness **(Tan *et al.*,2019; Limsui *et al.*, 2010).** Cancer treatment methods include surgery, radiotherapy, immunotherapy and chemotherapy **(Chalbatani *et al.*, 2019).** These techniques are individually useful in special cases and when linked, give more efficient treatment of the tumor.

Natural products from plants, the environment and microorganisms are leveraged for the purpose of fighting cancer**.**Roughly more than 60% of anti-cancer drugs are derived from these sources **(Cragg & Newman, 2009; Nobili *et al.*, 2019)**. In general, the term natural products refers to primary and secondary metabolic products, which are biologically active compounds with a low molecular weight less than 3000 Daltons produced by organisms that help them survival **(Kinghorn *et al.*, 2009– Zhang *et al.*, 2005).**

Natural products have the potential to inhibit cancer progression and reverse its progression **(Kaur *et al.*, 2011; Aravindaram& Yang, 2010).** Natural products are also an alternative solution to chemotherapy and its associated side effects such as heart failure, diarrhea, and others**.**Due to its high toxicity, it may lose specialization in treatment **(Tan *et al.*, 2015; Suter& Ewer, 2013).** Medical chemotherapy must be a specialist to get rid of a type of cancerous cell, but it cannot distinguish between normal and cancerous cells. However, most

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of the presently used anticancer treatments tend to destroy cancer and normal cells **(Ser *et al*., 2015).** Cancer chemoprevention is similarly essential as an interference in carcinogenesis. These can be obstructive agents that stop neoplastic process or defeating agents that inhibit the progress of cancer cells' malignant phenotype **(Tan *et al*., 2013; Surh, 2003).** Thus, it is a continuing work to search for highly specific and potent chemotherapy agents from substitute sources for example microorganisms.

Actinobacteria have been recognized as main sources of bioactive natural products as early as in the 1950s, for which about half of the secondary metabolites revealed, including enzymes, antibiotics, immunosuppressive, and anti-tumour agents, are formed by actinomycetes **(Dharmaraj, 2010; Kemung *et al*.,2018).** The well known representative genus of class Actinobacteria is the Streptomyces, which accounts for over 70% of commercially beneficial antibiotics **(Lee *et al*., 2 015; Pimentel-Elardo *et al*., 2010)**. Furthermore, it is notable that 80% of actinobacterial natural products documented previously are derived from the genus Streptomyces **(Dharmaraj, 2010).**

Recently the nanoparticle liposome has become important as a drug delivery vehicles. These carriers consist of a lipid bilayer that is a hollow spherical shape occupied by an aqueous phase. Therefore, any compound can be encapsulated inside the liposome in one of the two parts if it is hydrophilic inside and if it is lipophilic within the bilayer liposome **(Singh *et al*., 2019).** This formulation can encapsulate more than one drug at the same time, protecting the encapsulated compound from hydrolysis and degradation. In addition, targeting the surface proteins on the cells and ligand on lipid bilayer shell have addition functional allowing targeted entry of liposome into the cell, by ligand receptors target. These ligands attach to cell receptors that are over-expressed in convinced diseased cells, permitting entry of the drug through the cell membrane **(Eloy *et al*., 2014).**

Despite all of the above, studies regarding the potential biological effectiveness of actinomycetes metabolites as anti-cancer drugs and the discovery of new drugs are limited**.** In this respect, this study was developed as an attempt to discover the anticancer property from actinomycetes against Human colon adenocarcinoma cell lines.

2. Material and methods

2.1 Collection of Samples and Isolation of Actinomycetes

A 25 gm of agricultural soil sample was collected from a depth of 20 cm. It was placed in a sterile bag and transferred to the laboratory. For the purpose of isolating the Actinomycetes bacteria, the serial dilution method was implemented for the soil sample (1 gm of soil was diluted in 9 ml of distilled water and then 1 ml of this dilution was transferred. To 9 ml of distilled water and so on until the sixth dilution **(Williams et al., 1983).** 100 µl of the fourth and fifth dilutions were plotted on medium (starch nitrate agar (SNA)) prepared by (of 20 g/l starch, 1 g/L KNO3, 0.5 g/l K2HPO4, 0.5 g/l MgSO4.7H2O, 0.5 g/l NaCl, 0.01 g/l FeSO4, 15 g/l agar) and containing antifungal and bacterial agents (nystatin and naldixic acid) at 50 and 20 mg / l, respectively. The plates were then incubated at 30C for 7 days. The growth colonies were diagnosed morphologically and with some biochemical tests **(Aghamirian and Ghiasian, 2009; Reddy *et al.*, 2011)**.

2.2 Preparation of actinomycetes extract

Diagnosed isolates from Actinomycetes cultured on medium SNA At a degree of 28 c for a period of 7-14 days until the spors are completely formed. In a 250 ml flask containing medium ISP2 medium containing (4 g/l glucose, 4 g/l yeast extract, and 10 g/l malt extract,) was inoculated with spore suspension and incubated at 30 using a shaker incubator (150 rpm) for 15 days, the cells were separated using a centrifuge 5000 rpm and 4c and the extraction of cell biomass was with acetone and then the acetone was evaporated under the vacuum. The remaining water was extracted by acetyl acetate **(Shaaban *et al*., 2013).**

2.3 Separation and purification by HPLC technique

Shimadzu LC-6AD gradient pump, SPD-M20A prominence diode array detector, and HPLC 2D Chemstation software (Hewlett-Packard, Les Ulis, France). The chromatographic separation was performed with a reversed-phase column (Bio wide pare C18 ,25cm×4.6mm,5Mm supelco analytical column and Shim-pack prep-ODS 250×20 mml.D preparative column.).CBM-20A controller, DGU-20Adegaseos. FCR-10A, shimadzu fraction collector.

2.4 Development of separation protocol by analytical DAD-HPLC

The column temperature adjusted at 30 Cº at a flow rate of 1.0 ml/min to achieve the optimum resolution of the separation of many compounds .The injection volume was maintained at 20 µl of watery extract, the mobile phase had been employed to achieve the best separation condition was 50 % HPLC-grade acetonitrile

2.5 Fractionation protocol by preparative HPLC

The column temperature adjusted at 30 Cº at a flow rate of 10 ml/min to achieve the optimum resolution of the separation of compounds. The injection volume was maintained at 500 and 1000µl of extract.

2.6 Identification of chemical composition of the HPLC fractions using GC-MS

The collected fractions was analyzed by a coupled Varian gas chromatography/mass spectrometry (Perkin Elmer Auto XL GC, Waltham, MA, USA) equipped with a flame ionization detector to identify their chemical composition. The GC conditions were EQUITY-5 column (60 m 0.32 mm x 0.25 mm); H2 carrier gas; column head pressure 10 psi, the oven temperature was maintained initially at 70 C for 2 min, and then programmed from 70 to 250 C at a rate of 3 C/min. The ionization voltage was 70 eV and mass range m/z 39e400 amu. The identification of individual compounds was based on their retention times relative to those of authentic samples and matching spectral peaks available with the published data **(Iwasa *et al*., 2015).**

2.7 Nanoparticles- fractional mixture preparation

Nanoparticles Liposome Solution: It was ready prepared solution (according to Sigma Aldrich, Germany) and supplied in glass vial (0.4 mg) provided with nuclease free water (1ml) as a stock solution. The stock solution was diluted with adding distilled water in proportion of 100 µl of liposome: 900 µl D.W (4µg/ml). Each purified fractions at a concentration of 500 µg/0.5ml mixed with 0.5ml of Liposome. The proportion of 100 µl of liposome (Stock Solution) 900 µl D.W.(4µg/ml).

2.8 *In vitro* Anti-cancer Cytotoxicity

Each nano purified fractions were evaluated for their cytotoxicity using tissue culture technique. HT29 (Human colon adenocarcinoma)cell line was kindly provided by the National Cell Bank of Iran (NCBI), Pasteur Institute of Iran. Cells were

maintained in RPM1medium with 10% fetal calf serum, sodium pyruvate, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C and 5% CO2 till the cytotoxicity bioassay was carried out. The potential cytotoxicity of nano purified fractions was tested using the method of Alley **et al. (1988)**. Briefly, 100 cells/well were plated onto 96-well dishes overnight before the treatment with the tested compounds to allow the attachment of cells to the wall of the plate. Different concentrations of each tested compound (0, 15.6, 31.25, 62.5, 125, 250 and 500µg/ml) were added to the cell monolayer and triple wells were used for each individual dose. Monolayer cells were incubated with the tested agent(s) for 48 h at 37°C and 5% CO2. At the end of the incubation period, Crystal violet (C.V) assay was used to determine the optical density of the cell growth in each well of the microtiter plate, by using plate reader. After the end point of cytotoxicity assay, the maintenance medium with the test substance was discarded out and the wells washed with 100 µl of cold PBS by automatic pipette. Then the cell cultures were fixed with 10 % buffered formalin for 20 min at room temperature. Fixative solution was discarded and 100 µl of 0.1 % aqueous CV solution was added to each well. The samples were incubated at room temperature for 20 min with gentle shaking. After that the plates were washed by submersion in flowing tap water for 15 min. The plates were allowed to dry in the air and the absorbance was read at 570nm by a microplate reader **(Castro-Garza *et al.*, 2007).**The percentage of inhibition was calculated according to the following equation: **(Chiang *et al.*, 2003)**

Inhibition Rate (I.R) % = (optical density of control wells - optical density of test wells) / (optical density of control wells) X 100.

The relation between surviving fraction and compound concentration was plotted to get the survival curve of each tumor cell line and the IC50. The concentration of an agent that causes a 50% growth inhibition, for each tested agent using each cell line was obtained from the survival curve **(Skehan *et al.*, 1990).**

3. Results

3.1 Identification of Actinomycetes isolates

The colonies growing on a SNA medium were morphologically identified where the colonies were well-growth and had a gray color, not producing dyes in the medium. The results of the biochemical tests indicated that isolates were amylase, catalase, and gelatinase producing isolates and non-lipase producing, non H2S production and consuming urea, while the carbon consumption test indicated the isolates' ability to consume starch, glucose and sucrose (Table 1).

Table 1 Morphological growth and biochemical tests of Actinomycetes isolates

Characters	Results
Growth on SNA medium	Gray color colony, no dyes production
Amylase production	+
Catalase production	+
Gelatinase production	+
Lipase production	-
H2S production	-
Urea decomposition	+
Starch utilization	+
Glucose utilization	+
Sucrose utilization	+

3.2 Fractionation by preparative HPLC

The results of preparative HPLC revealed that 4 fractions were collected with desired amounts of each compound when using fraction collector in depend on mobile phase system in analytical HPLC with (50 % HPLC-grade acetonitrile) at 254 nm and cycling up was employed to increase the separation efficiency, each fraction were collected at specific retention time (min) (Table 2).

Table 2 Fraction number and retention time (min) used preparation HPLC with separation system 50 % HPLC-grade acetonitrile

Fraction no.	Retention time
1	6.5
2	7.2
3	7.8
4	9.5

3.3 Chemical composition of the HPLC fractions using GC-MS

The chemical composition of four fractions which analysis by GC/MS showed the identification of many components, representing the major components in the each fractions arranged based on the retention time and area were showed in (Table 3) and (Figure 1).

Table 3 The chemical composition of four fractions

Fraction no.	Chemical composition	Rt.	Area %
1	Hexadeconic acid	10.362	52.42
	13-Docosenamide	32.256	7.003
	n-pentadecanoic acid ,trimethylsilylsilyl ester	47.808	9.52
2	Octadecanoic acid,ethyl ester	28.054	26.75
	2-benzothiazolecarboxaldehyde	5.910	2.55
	1-butamine,N-nitro-N-propyl	45.036	14.13
3	Trisiloxane ,1,1,3,3,5,5-hexamethyl	5.993	1.69
	Heaxdeconic acid,ethyl ester	22.013	44.56
	Octadecanoic acid,ethyl ester	27.902	32.20
4	Trisiloxane ,1,1,3,3,5,5-hexamethyl	6.014	26.47
	Butanedioc acid,dimethoxy-diethyl ester	45.054	112.72
	Fumaric acid,isopropyl tetradecylester	46.217	13.39

3.4 *In vitro* Anti-tumor Cytotoxicity

The results in (figure 2) showed that all four nano purified fractions were applied on HT 29 colon cancer cells and exhibited significantly differences compared with control treatments of inhibition cells number. Where the highest rate of inhibition of cancer cells was about 81.5, 89, 93.8 and 90.1%) for the nanofractions 1, 2, 3 and 4, respectively, which subjected with concentration 500 mg/ml. When using the half dilution series, the inhibition percentage decreased gradually with dose dependent response decreasing, And these data were used to calculate the values of IC50 (the inhibitory value of half the number for all nanofractions. the application of concentration with inhibition value and solved the equation to IC50 value were gained, which were (151.4, 16.4, 16.6 and 43.8 µg/ml) to four nano fractions respectively (Figure 2 and 3).

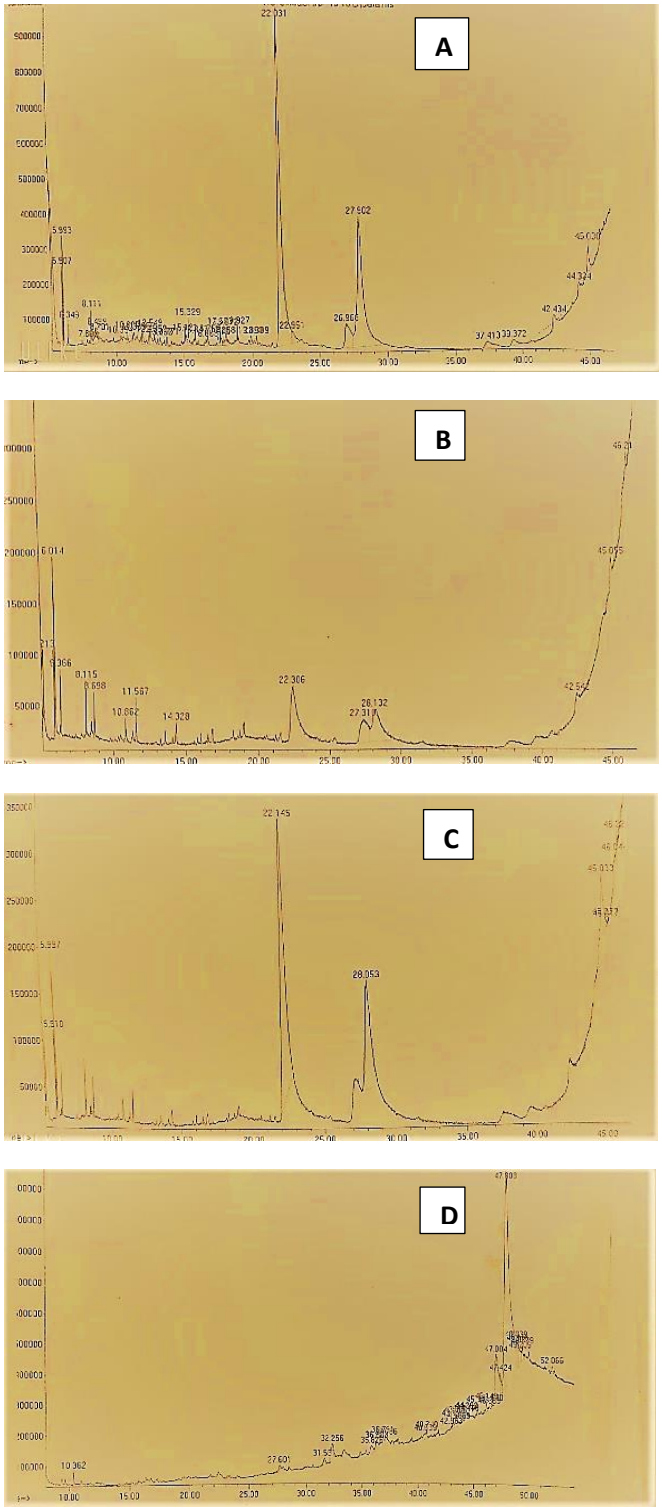

Figure 1 GC-MS chromatograph of Actinomycetes purified fractions (A-fraction 1, B-fraction 2, C-fraction 3and D-fraction 4)

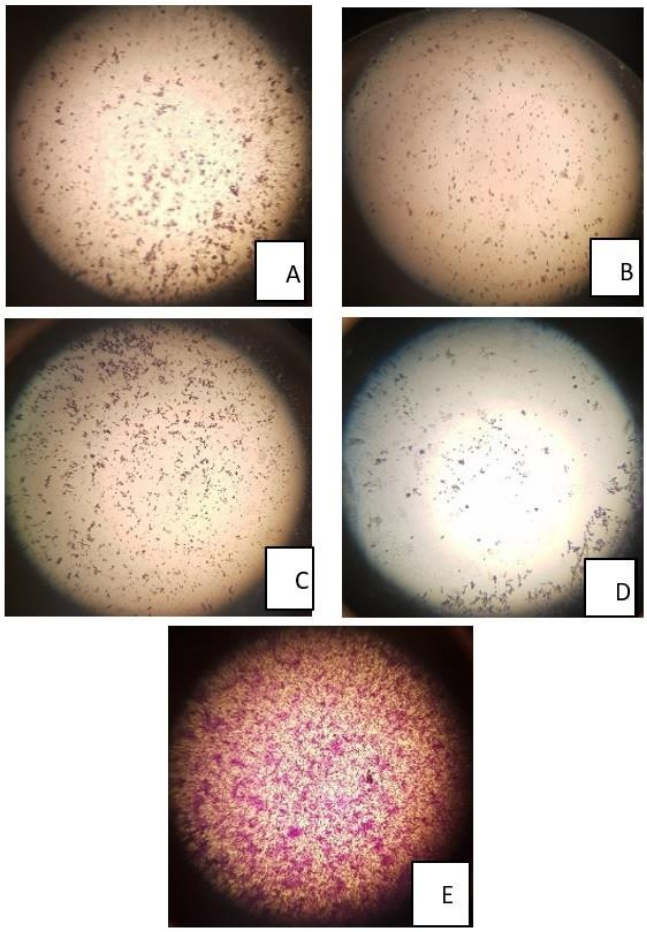

Figure 2 *In vitro* anti-cancer cytotoxicity assay of HT 29 colon cancer cell line subjected to A) fraction 1 nanoparticle 500 µg/ml. B- fraction 2nanoparticle 500 µg/ml c-fraction 3 nano particle500 µg/ml D- fraction 3nanoparticle 500 µg/ml and E-control HT 29 colon cancer cell line .

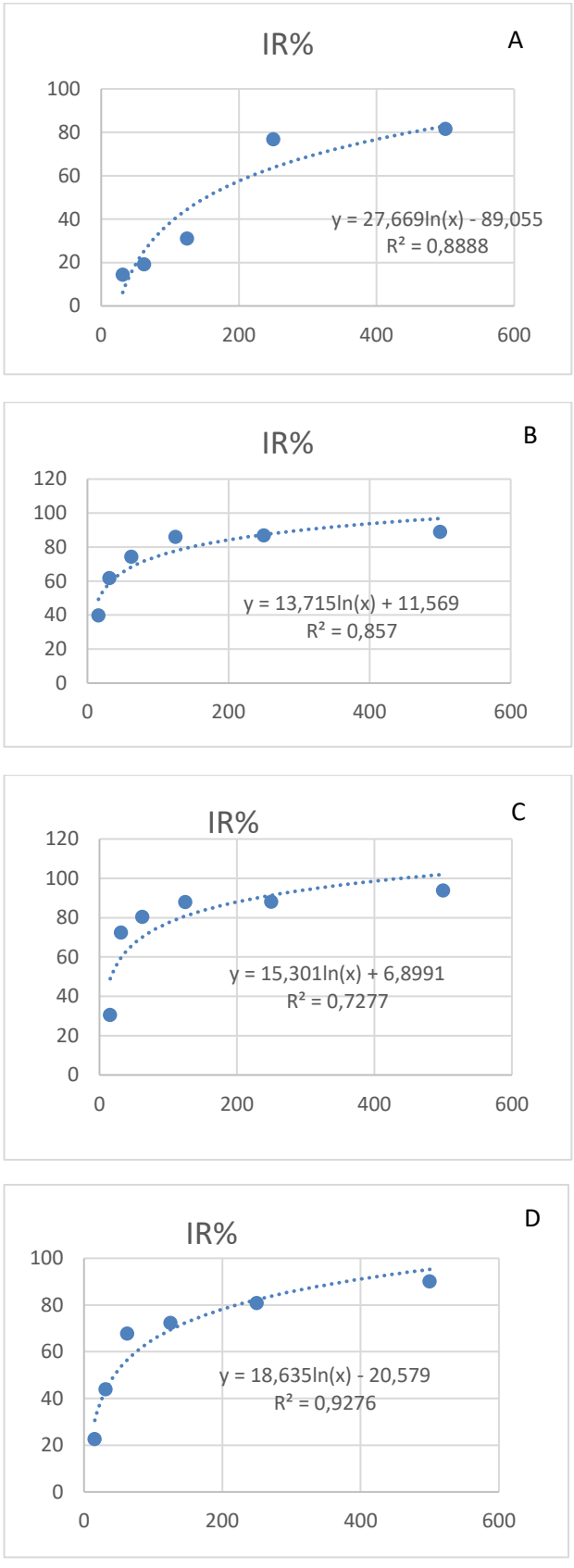

Figure 3 Dose response curve of growth inhibition of HT 29 colon cancer cell line when subjected to 1,2,3 and 4 nanoparticle fractions of actinomycetes (A,B,C and D)respectiveily presented by plotting of concentration versus IR% values.

4. Discussion

Actinomycetes is a Gram-positive, aerobic bacterium that is belonging to the order actinomycetales characterized by having an aerial mycelium. It is the most common filamentous organisms in the soil, and it is responsible for the smell of the earth, which indicates the vitality of the soil. It has a major role in the recycling of organic matter **(Bhatti and Bhat, 2017).**Actinomycetes is widespread in various habitats and participates in important processes, as it not only can live in harsh soil conditions such as lack of moisture and high salinity, but it stimulates plant growth. **(Hamdali *et al.*, 2008).**

In Georgia in the United States I refer to an example of filamentous bacteria prevalent in Pasture and cultivated soils (Lauber et al., 2009).In addition, **Burck *et al.* (2003)** indicated that Actinomycosis is the most common bacterial community in agricultural soils compared with forest soils when these soils were analyzed and compared in different countries. Moreover, he determined that actinomycetes increases after the transfer of lands from forest to agricultural **(Burck *et al.*, 2003; Fierer *et al.*, 2009)**.

Due to their biological importance and effectiveness, the secondary metabolic products of microbes have captured the interest of researchers, especially those that have an impact on human health. The biosynthesis of these products through engineering and biotechnology has shown significant benefits from conventional biomass extraction methods. Many types of soil bacteria produce unique secondary metabolic products that play important roles in many biological activities, the most important of which is Actinomycetes, which plays an important role in the manufacture of medicinal and pharmaceutical preparations due to the ability and effectiveness of these metabolic products and in various chemical compositions and Biological activities. Thousands of bioactive compounds have been isolated, diagnosed and developed from many different drugs to treat a wide range of human diseases, their poultry and their agriculture sectors **(Castillo *et al.*, 2002; El-Shatoury *et al.*, 2009).**

Actinomycetes is also a potential source of many metabolic by-products, antibiotics and other active compounds , It has a latent genetic potential to produce 10-20 secondary metabolites **(Bentley *et al.*,2002; Sosio *et al.*,2000).**There are evidence indicated it is a source of 75% of the compounds known as antibiotics, **(Nolan & Cross,1988; Thakur *et al.*,2009).** In addition to the antibacterial and antifungal the Streptomycetes produces anticancer drugs such as driamycin and the immunosuppressant tacrolimus **(Hopwood, 2007)** and contributes approximately 70% of the described metabolic products of filamentous bacteria **(Zengler *et al.*,2005).** Streptomycetes and other filamentous bacteria are useful sources of secondary metabolic products with numerous biological activities that may eventually be applied to the creation of effective anti-cancer agents and other beneficial pharmaceutical compounds **(Bibb, 2005).**

The chemical analysis of the purified fractions from the actinomycetes extract indicated the predominance of several compounds that may have the inhibition effect of cancer cells. Among them are compounds of the type of furan that **(Nguyen *et al.*, 2020)** indicated their anti-cancer ability when applied to cell lines of type (AGS, HCT116, A375M, U87MG, and A549) with IC50 values of 40.5, 123.7, 84.67, 50, and 58.64 µM, respectively.

It was also observed that Pentadecanoic acid has the effect of selective toxicity in MCF-7/SC comparison with parental cells. In addition, pentadecanoic acid inhibits the progressive and proliferative ability of cancer cells as indicated **(Nguyen *et al.*, 2020)** This is due to the ability of pentadecanoic acid to increase the gene expression of cancer cells to produce cleaved caspase-3, -7, -8, associated with the process of programmed cell death,

as increased production leads to apoptosis of cancer cell **(McIlwain *et al.*, 2015).**

A recent study demonstrates that heptadecanoic acid can exert anti-cancer effects on lung carcinoma cell line, emphasizing the efficacy of fatty acids in targeting human lung cancer cells **(Xu *et al.,* 2019).**

Also some researches showed that Fumaric acid used for inhibiting the solid growth of Ehrlich tumor in mice, was found to reduce markedly the growth and viability of Ehrlich, MH134, and L1210 mouse tumor cells in culture at concentration of 0.3 approximately 1.2 mg/ml**.(Kuroda and Akao, 1981) .**

There is no doubt that the use of nanocomposites as a catalyst in increasing target identification and ensuring intracellular access to drugs has been referred to in many studies one important example Nanoscale drug delivery systems using liposomes and nanoparticles are emerging technologies for the rational delivery of chemotherapeutic drugs in the treatment of cancer. Their use offers improved pharmacokinetic properties, controlled and sustained release of drugs and, more importantly, lower systemic toxicity **(Malam *et al.,* 2009).**

5. Conclusion

This study showed that the actinobacteria extract has very high efficacy against cancer cell lines of the type of colon cancer, as the compounds purified from the extract by HPLC, the chemical analysis of them by GC-mass showed they contain compounds that act to inhibit the cancer cells in addition to the increase in the effectiveness of these compounds. From its combination with liposomes nanoparticles that served to deliver the active substance into the cancer cell and destroy it.

Declaration of interest

The authors report no conflicts of interest.

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Evaluation of Heavy Metal Contents and Potential Human Health Risk Assessment of Selected Canned Sardines Fish Sold in Yenagoa, Nigeria

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Abstract

Canned sardine fish is consumed regularly in all countries. The levels of heavy metals which are present in the environment could constitute a hazard to food security and public health. These can be accumulated in aquatic animals such as fish. In this study, selected heavy metals: Copper (Cu), Nickel (Ni), Chromium (Cr), Zinc (Zn), Cadmium (Cd), Manganese (Mn), Lead (Pb) and Iron (Fe) were evaluated in Sardines that are commonly consumed in Nigeria. Eight different brands of canned sardines were purchased in Yenagoa and were taken to the Laboratory for heavy metal analysis. Standard wet digestion procedure was adopted for sample preparation while Atomic Absorption Spectrophotometer (AAS) technique was adopted for metal analysis. The results show that heavy metal concentrations of Fe in the samples were above World Health Organization (WHO) and United States Environmental Protection Agency (USEPA) permissible limits, while Zn was below the permissible limits. However, Pb, Cu, Cd, Cr, Ni and Mn were below detectable limits in all the samples. Daily intake of metal (DIM) values in the sardine samples for Zn and Fe were significantly lower than the recommended daily intake and the upper tolerable daily intake levels. The Health risk index (HRI), Target hazard quotient (THQ) and Hazard Index (HI) for Zn and Fe in this study were less than 1(<1) for both adult and children, therefore, the canned sardines does not pose a potential human health risk concern for the consumers. However, the high Fe concentrations in all the sardine samples may cause public health concern as they are above WHO and USEPA tolerable limits.

1. Introduction

Heavy metals are natural components of the Earth's crust. They cannot be degraded or destroyed. To a small extent they enter our bodies via food, drinking water and air. As trace elements, some heavy metals are essential to maintain the metabolism of the human body. At higher concentrations these heavy metals can lead to poisoning. Heavy metal poisoning could result, for instance, from drinking-water contamination, high ambient air concentrations near emission sources, or intake via the food chain. A group of heavy metals are classified as non-essential, because they have no biological, chemical and physiological importance in man. After acute and chronic exposures, they causes a variety of adverse health effects to humans such as dermal changes, respiratory, pulmonary, cardiovascular, gastrointestinal, haemalogical, hepatic, renal, neurological, developmental, reproductive, immunologic, genotoxic, mutagenic, and carcinogenic effects **(Mandal & Suzuki, 2002)**. Some heavy metals, such as cadmium and lead, injure the kidney and cause symptoms of chronic toxicity, including impaired organ function, poor reproductive capacity, hypertension, tumors, and hepatic dysfunction **(Abou-Arab et al., 1996)**. Sub-lethal effects of heavy metals are of concern as they accumulate and are transferred through the food-chain to humans **(Yilmaz & Yilmaz, 2007)**.

The human health risk assessment is the process requires identification, collection, and integration of information on the toxins and chemicals health hazards, exposure of human to the chemical and relationships between exposure, dose and adverse health effects in polluted environmental. On the other hand, a human potential health risk assessment includes hazard identification, dose-response assessment, exposure assessment and risk characterization steps **(Sobhanardakani, 2017)**.

In recent years, concern about food quality has increased, particularly in those foods at risk of containing toxic elements and compounds that represent a risk to human health, such as persistent organic pollutants (POPs) and heavy metals. There is increasing concern about the quality of canned foods in several parts of the world. The determination of toxic elements in food has prompted studies on their toxicological effects. Heavy metals pose a common threat to all organisms in the ecosystem by forming an ever increasing accumulation through food chain. Humans may be exposed to harmful nonessential elements such as arsenic, silver, lead, mercury, cadmium, and nickel mainly through drinking water, consumption of fresh and processed foods and through occupational exposures **(Ikem & Egiebor, 2005)**.

Fish consumption has increased simultaneously with the growing concern of their nutritional and therapeutic benefits. In addition to its important source of protein, fish typically have

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rich contents of essential minerals, vitamins and unsaturated fatty acids **(Medeiros et al., 2012)**. The American Heart Association recommended eating fish at least twice per week in order to reach the daily intake of omega-3 fatty acids **(Kris-Etherton et al., 2002)**. However, fish are relatively situated at the top of the aquatic food chain; therefore, they normally can accumulate heavy metals from food, water and sediments **(Yilmaz et al., 2007; Zhao et al., 2012)**. The content of toxic heavy metals in fish can counteract their beneficial effects; several adverse effects of heavy metals to human health have been known for long time **(Castro-Gonzalaz & Mendez-Armenta, 2008)**. This may include serious threats like renal failure, liver damage, cardiovascular diseases and even death **(Al-Busadi et al., 2011; Rahman et al., 2012)**. Therefore, many international monitoring programs have been established in order to assess the quality of fish for human consumption and to monitor the health of the aquatic ecosystem **(Meche et al., 2010)**.

In the last few decades, the concentrations of heavy metals in fish have been extensively studied in different parts of the world **(Elnabris et al., 2013)**. Most of these studies concentrated mainly on the heavy metals in the edible part (fish muscles). However, other studies reported the distribution of metals in different organs like the liver, kidneys, heart, gonads, bone, digestive tract and brain. This study was aimed at evaluating heavy metal contents and potential human health risk assessments of selected canned sardines fish sold in Yenagoa, Nigeria.

2. Material and methods

2.1 Sample Collection

Eight (8) samples of canned sardines' fish were purchased at Yenagoa, Bayelsa State, Nigeria and were taken to the laboratory for toxic metal analysis. The samples were designated as; SR, TS, OA, DR, LJ, NA, CA and VA.

2.2 Samples Preparation and Procedure

Wet digestion method was used in the preparation of the Sardine samples for heavy metal analysis. 5 ml of analytical unit (sample) was weighed into digestive tube and 20 ml of digestion acid at ratio 1: 3: 1: 1: (HNO_3 + H_2SO_4 + HCl + $HClO_4$) was added. This was latter digested using FOSS TECATOR Digester Model 210 at 250°C for 1 hour at the first instance and continued until a clear solution was obtained in a fume cupboard. The clear solution was filtered into a 100 ml volumetric flask and completed to the mark with de-ionised water.

2.3 Determination of toxic metals

All digested samples were analyzed in triplicate using Atomic Absorption Spectrophotometer (Buck 210). Standard for each element under investigation was prepared in mgl/100g and the limit standard concentration for each element was adhered to according to the BUCK Scientific instruction and the results obtained were compared with World Health Organization standards for the metal limits for human consumption.

2.4 Quality assurance protocol and statistical analysis

Appropriate quality assurance procedure and precautions were carried out to ensure reliability of the results. Samples were carefully handled to avoid contamination. Glassware and sample containers were soaked in 1 mol/L HNO_3 for 48 h and rinsed with ultrapure water and the reagents were of analytical grade. Precision and accuracy of the analytical procedure was also investigated by carrying out recovery experiments. Accuracy of the digestion procedures was verified by examination of the recovery data, spiking analyzed samples with aliquots of metal standards and then reanalyzing the samples. The percentage recovery was greater than 95% with the percent relative standard deviations less than eleven, indicating good accuracy and precision. The results were expressed as mean ± standard (SD) using SPSS Statistic 17.0.

2.5 Health risk assessment

The potential health risks of heavy metal consumption through sardines were assessed based on the daily intake of metal (DIM) **(Chary et al., 2008)**, health risk index (HRI) **(Jan et al., 2010)**, and the target hazard quotient (THQ) **(Wang et al., 2005; Storelli, 2008)**. The daily intake of metals (DIM) was calculated to averagely estimate the daily metal loading into the body system of a specified body weight of a consumer. This will inform the relative availability of metal. This does not take into cognizance the possible metabolic ejection of the metals but can easily tell the possible ingestion rate of a particular metal. The estimated daily intake of metal in this study was calculated based on the formula below:

$$DIM = \frac{Cmetal \times Cfactor \times Cfood\ intake}{Body\ average\ weight} \quad (1)$$

Where,
C metal is the heavy metal conc. in sardine (mg/kg),
C factor is the conversion factor,
C food intake is the daily intake of sardine.

The conversion factor of 0.085 was used in this study, daily sardine intake of 65 g/day for adult and 28.35 g/day for children, while the average body weight used was 65 kg for adult and 24 kg for children in this study **(Oguntona, 1998)**.

The health risk index (HRI) was calculated using the formula below:

$$HRI = \frac{DIM}{RFD} \quad (2)$$

The THQ was calculated using the formula below:

$$THQ = \frac{EF \times ED \times FIR \times C}{RFD \times WAB \times TA} \times 10^{-3} \quad (3)$$

Where:
EF is the exposure frequency (350 days/year)
ED is the exposure duration (60 years, equivalent to the average lifetime of the Nigerian population for adult and 20 years for children were adopted)
FIR is the food ingestion rate (sardine consumption values is 65 g/person/day) **(Oguntona, 1998)**;
C is the metal concentration in the sardine (mg/kg);
RFD is the oral reference dose (Zn and Fe values were 0.300 and 0.700 mg/kg/day, respectively) **(USEPA IRIS, 2006).**
WAB is the average body weight (65 kg for adults and 24 kg for children was adopted in this study) **(Oguntona, 1998)** and
TA is the average exposure time for non-carcinogens (ED x 365 days/year). If the THQ value is greater than 1, the exposure is likely to cause obvious adverse effects.

Calculation of hazard index: Hazard index is used to evaluate the potential risk to human health when more than one toxic metal is involved. Hazard index was calculated as the sum of target hazard quotients (THQs) **(Abou-Arab, 2001)**. Since different

pollutants can cause similar adverse health effects, it is often appropriate to combine THQs associated with different substances **(Al-Jassir et al., 2005)** as seen in equation 4.

$$HI = \Sigma THQ\ (THQ1 + THQ2 + THQ3 \ldots THQn \quad (4)$$

3. Results

3.1 Heavy Metal contents

Heavy metal contents in the sardine samples are shown in Table 1. The highest mean value of Zn was TS (0.14±0.001 mg/kg) and lowest was VA (0.03±0.001 mg/kg). Highest Fe mean value was 2.67±0.001 mg/kg (TS) and lowest was NA (1.34 ±0.001 mg/kg). Meanwhile, Cu, Ni, Pb, Cd, Mn and Cr were below detectable limits.

3.2 Potential health risk assessment

The DIM values of Zn and Fe from the sardine samples were below the permissible tolerable daily intake limits as shown Tables 2 and 3 for both adult and children. Health risk index, Target hazard quotient (THQ) and hazard index (HI) values were less than 1 (Tables 2 and 3).

Table 1 Heavy Metal Contents of Various Sardines Sold in Yenagoa

Sample Code	Heavy metals (mg/kg)							
	Cu	**Zn**	**Ni**	**Pb**	**Cd**	**Fe**	**Mn**	**Cr**
SR	BDL	0.11±0.001	BDL	BDL	BDL	1.45±0.001	BDL	BDL
TS	BDL	0.14±0.001	BDL	BDL	BDL	2.67±0.001	BDL	BDL
OA	BDL	0.05±0.001	BDL	BDL	BDL	1.93±0.001	BDL	BDL
DR	BDL	0.09±0.0009	BDL	BDL	BDL	2.30±0.001	BDL	BDL
LJ	BDL	0.06±0.001	BDL	BDL	BDL	1.90±0.001	BDL	BDL
NA	BDL	0.05±0.001	BDL	BDL	BDL	1.34±0.001	BDL	BDL
CA	BDL	0.07±0.001	BDL	BDL	BDL	1.70±0.001	BDL	BDL
VA	BDL	0.03±0.001	BDL	BDL	BDL	1.62±0.001	BDL	BDL
USEPA	1.30	5.00	0.02	0.015	0.005	0.3	0.05	0.05
WHO	2.0	5.00	0.02	0.01	0.003	0.3	0.10	0.05

BDL<0.001; BDL: Below Detectable Limit, WHO: World Health Organization, USEPA: United States Environmental Protection Agency, Values are Mean±SD triplicate determination

Table 2 Results of Daily intake of metal, Health risk index, Target hazard quotient and Hazard index of heavy metals in sardine for adults (65kg)

Sample code	DIM		HRI		THQ		HI
	Heavy Metal		Heavy metal		Heavy metal		
	Zn	**Fe**	**Zn**	**Fe**	**Zn**	**Fe**	
SR	0.0041	0.054	0.014	0.08	1.53E-4	8.66E-4	1.02E-3
TS	0.0052	0.099	0.017	0.14	1.95E-4	1.59E-3	1.79E-3
OA	0.0019	0.072	0.006	0.10	6.97E-5	1.15E-3	1.22E-3
DR	0.0033	0.085	0.011	0.12	1.25E-4	1.37E-3	1.49E-3
LJ	0.0022	0.070	0.007	0.10	8.36E-5	1.14E-3	1.22E-3
NA	0.0019	0.049	0.006	0.07	6.97E-5	8.01E-4	8.71E-4
CA	0.0026	0.063	0.009	0.09	9.75E-5	1.02E-3	1.11E-3
VA	0.0011	0.060	0.004	0.09	4.18E-5	9.68E-4	1.01E-3

DIM; Daily intake of metal; HRI; Health risk index; THQ; Target hazard quotient; HI; Hazard Index

Table 3. Results of Daily intake of metal, Health risk index, Target hazard quotient and Hazard index of heavy metals in sardine for Children (24kg)

Sample code	DIM		HRI		THQ		HI
	Heavy Metal		Heavy metal		Heavy metal		
	Zn	**Fe**	**Zn**	**Fe**	**Zn**	**Fe**	
SR	0.011	0.145	0.037	0.21	4.15E-4	2.34E-3	2.75E-3
TS	0.0141	0.268	0.047	0.38	5.28E-4	4.32E-3	4.85E-3
OA	0.0050	0.194	0.016	0.28	1.88E-4	3.12E-3	3.31E-3
DR	0.0090	0.231	0.030	0.33	3.39E-4	3.72E-3	4.06E-3
LJ	0.0060	0.191	0.020	0.27	2.26E-4	3.07E-3	3.29E-3
NA	0.0050	0.135	0.016	0.19	1.88E-4	2.16E-3	2.35E-3
CA	0.0070	0.170	0.023	0.24	2.64E-4	2.75E-3	3.01E-3
VA	0.0030	0.163	0.010	0.23	1.13E-4	2.62E-3	2.73E-3

DIM; Daily intake of metal; HRI; Health risk index; THQ; Target hazard quotient; HI; Hazard Index

4. Discussion

4.1 Heavy metal contents

Some factors, such as water chemistry, duration of exposure of fish to contaminants in water, concentrations of contaminants in water column, feeding habit of fish, contamination of fish during handling and processing, quality of canned fish and shelf life of canned fish can affect in the level of contaminants in fish. However, the metal levels in canned fishes is influenced by the pH of the canned product, oxygen concentration in the headspace, the quality of the lacquer coatings of canned products, quality of coating and also storage place **(Tahán et al., 1995; Hosseini et al., 2013)**. The metals accumulation varies greatly between both fish species and/or fish tissues. Generally, fish could translocate the large quantities of toxic heavy metals in the liver, gill, and also muscle tissues **(Sobhanardakani et al., 2012)**. Contaminants in fish can pose a health risk to the fish

themselves, to their predators, and to humans who consume them **(Burger & Gochfeld, 2005)**.

Iron is an essential mineral and is the most abundant transition element, and probably the most well-known metal in biologic systems especially plays an important role in the human physiology. Iron deficiency causes anemia, reducing cognitive function and also physical work capacity. Whereas, high intake of this element may be the cause of organ failure **(Mol, 2011; Hussein & Khaled, 2014; Stancheva et al., 2014; Wheal et al., 2016)**. All the Fe concentrations in the samples analyzed were above USEPA and WHO recommended permissible limits of 0.3 mg/kg. These high Fe concentrations might be as a result of the cans used for packaging the fish. These findings are in agreement with the reports of **Khalid & Samir, (2016)**.

Zinc is known to be involved in most metabolic pathways in humans and zinc deficiency can lead to loss of appetite, and other health problems. Zinc is widespread among living organisms, due to its biological significance. The maximum zinc level permitted for fish is 50mg/kg according to Food Codex. The UK required nutritional intake (RNI) ranges set by COMA for zinc are 5.5-9.5 mg/kg/day for adult males and 4.0- 7.0 mg/day for adult females. Zinc is an essential element with a recommended daily allowances ranging from 5 mg for infants to 15 mg for adults. Too little zinc can cause health problems, but too much zinc is also harmful. Harmful health effects generally begin at levels in the 100 to 250 mg/day range **(RAIS, 2007)**. The present results showed that all the Zn concentrations in the samples analyzed were below USEPA and WHO recommended permissible limits. This result is in accordance with the findings of **Rustu, (2016)**, while Cu, Ni, Pb, Cd, Mn and Cr were below detectable limits.

4.2 Health risk assessment

To assess the health risk of the consumers of both adult and children due to heavy metal intake from sardines consumption, the daily intake of metals (DIM), health risk index (HRI), target hazard quotient (THQ) and hazard index (HI) were calculated from equations 1, 2, 3 and 4 respectively and the results are presented in Tables 2 and 3. The DIM results both for adult and children were compared with the recommended daily intake of metals and the upper tolerable daily intake level (UL) established by the Institute of Medicine for people between the ages of 19 to 70 years **(FDA, 2001; Garcia-Rico, 2007)**. It is very clear that daily intake of metals in sardine samples for Zn and Fe are significantly lower than the recommended daily intake of Zn and Fe (8 mg day^{-1} $person^{-1}$) and the upper tolerable daily intake level (UL) of Zn (40 mg day^{-1} $person^{-1}$) and Fe (18 mg day^{-1} $person^{-1}$) **(USEPA, 2010)**. The HRI for Zn and Fe from this study were less than 1 (HRI < 1) for both adult and children. Generally, HRI < 1 means that the exposed population is safe of metals health risk while HRI > 1 means the reverse **(Khan et al., 2008)**. The consumers are therefore at no risk of Zn and Fe was also reported by **Tsafe et al. (2012)**.

The THQ is a ratio between the measured concentrations and the oral reference dose, weighted by the length and frequency of exposure, amount ingested and body weight **(Tsafe et al., 2012)**. The parameter defines the exposure duration and the risk with that period. The THQ values of Zn and Fe due to sardine consumption for the populace (adults and children) of the study are shown in Tables 6 and 7. In this study, the THQ in Zn and Fe is far less than 1 in the entire sardine samples for both adult and children, therefore, it does not pose health risk concern. Meanwhile, Hazard Index (HI) is the calculation which shows when a population is at risk. From the results in the present study, it was observed that the combined HI values for all the samples under study were less than (<) 1 which indicates that there are no potential health risk to those consuming these sardines.

5. Conclusion

Heavy metal contamination in canned foods has been an important topic. Facility modernization and quality manufacturing are required to prevent heavy metal contamination in sardines and other canned products and thus the possible health hazards to the consumer. A long-term and/or excessive consumption of foods containing heavy metals above the tolerance levels has a hazardous impact on human health. Since canned foods are widely consumed, they contribute a large fraction to the heavy metals intake and, therefore, strict control of these elements is advisable. For this reason, the steps in all processes must be monitored for preventing the contamination by heavy metals. Also, this present study confirms the fact that all the brands of sardines fish analyzed were safe for human consumption as there was no potential health risk to consumers. However, the shelf life of these brands needs to be reduced to avoid oxygen intake by rusted cans and result to leaching of alloyed materials into the food.

Declaration of interest

The authors report no conflicts of interest.

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Comparative Phytochemical and Antimicrobial Properties of Two Cultivars of *Catharanthus roseus* L. {G.} Don on *Escherichia coli* and *Candida albicans*

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Abstract

Medicinal plants have served as sources of medicine to treat and suppress the diseases, because many pathogens are gaining resistance to the current synthetic drugs. In addition, high cost and adverse side effects are commonly associated with popular Synthetic drugs. Therefore, there is need for continuous search for new drugs in order to overcome this emerging resistance. Plants synthesize bioactive compounds which are of great potential in agriculture, antimicrobial and anti-insect activity. The concentration of bioactive compounds in each plant species depends on the environmental conditions, age of the plant, relative humidity of harvested materials and method of extraction. Little is known on the phytochemical and antimicrobial potential of Alba and Rosea cultivars of *Catharanthus roseus* ethanol extracts. The leaves of Alba and Rosea cultivars were investigated for their phytochemical and antimicrobial properties. The study was conducted at Maseno University, Kenya. Plant Leaves were collected around Maseno University. Leaves of Alba and Rosea cultivars of *Catharanthus roseus* were air-dried in the shade, thereafter crushed into powder and ethanol extraction done using the Rotary evaporator. Antimicrobial activity of the pathogenic microorganisms was *Candida albicans* and *Escherichia coli*. The paper disc diffusion method was used for antimicrobial tests. Different concentrations of ethanol leaf extracts which consisted of 2.5, 5and 7.5 mg/mL with three replications. Sterile water was used a control. The data on growth inhibition were subjected to analysis of variance (ANOVA) using SAS statistical package. Treatment means were separated and compared at p = 0.05. Phytochemical analysis revealed the presence of tannins, flavonoids, terpenoids, saponins, alkaloids and phenols in the leaf extract except steroids and glycosides. The ethanol leaf extracts were active against *Candida albicans* and *Escherichia coli*. Alba leaves extracts showed higher inhibitory zones compared to Rosea leaves. The observed differences in antimicrobial activity could be due to differences in cell wall synthesis, structure and composition. The results of present study further confirm the use of these plants traditionally for the treatment of different ailments.

1. Introduction

The use of plants for medicine has been practiced for many years (**Kokwaro, 2009; Musyimi *et al.*, 2008**). Man has used various parts of plants in the treatment and prevention of various ailments (**Mohammed *et al.*, 2011**). Plants produce secondary metabolites such as alkaloids, cyanogenic glycosides, glucosinolates, flavanoids, saponins, steroids and terpenoids (**Shalini and Sampathkumar, 2012**). Tannins possess anti-fibrotic effects (**Chuang *et al.*, 2011**). These bioactive compounds are of great potential in agriculture, antimicrobial and anti-insect activity (**Emitaro *et al.*, 2020b**). Endophytes synthesize bioactive compounds or their precursors which help them protect the host plants against pathogens (**Emitaro *et al.*, 2020a**). Previous studies have shown that endophytic microbial communities within medicinal plants have a great potential as producers of novel bioactive compounds and hence high potential for agricultural and pharmaceutical (**Köberl *et al.*, 2013; Rai *et al.*, 2014**). Tanshinones have diverse pharmacological activities such as anticancer, antidiabetes, cardioprotective effects and neuro-protective activity (**Teimoori-Boghsani *et al.*, 2020**). Microbial infections pose a health problem throughout the World, and plants are a possible source of antimicrobial agents (**Burapadaja and Bunchoo, 1995; Adenisa *et al.*, 2000**). Medicinal plants contain active principles which can be used as an alternative to cheap and effective herbal drugs against common bacterial infections. The curative properties of medicinal plants are attributed to the presence of various phytochemicals (**Sheeraz *et al.*, 2013**). The concentration of bioactive compounds in each plant species depends on the environmental conditions, age of the plant, relative humidity of harvested materials and method of extraction (***Emitaro et al.*, 2020a**). Phytochemicals distribution patterns in plants may differ within and between geographical locations due to differences in environmental conditions. Phytochemicals have the ability to protect humans against various diseases (**Rumzhum *et al.*, 2012**). Phytochemicals such as anthraquinones, tannins, terpenoids, and glycosides have antimicrobial activities and antioxidant properties (**Zheng *et al.*, 2001; Tiwari *et al.*, 2011; Benhammou *et al.*, 2013**). The

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medicinal value of a plant depends on the chemical constituents in it that produce definite physiological action on the human body (**Aiyelaegbe and Osamudiamen, 2007; Musyimi *et al.,* 2008**). Screening of herbs for pharmacological activities and phytochemical constituents is one of the active fields of research round the world today (**Khair-ul-Bariyah *et al.,* 2012; Sheeraz *et al.*, 2013**). *Catharanthus roseus* (L.) is an important medicinal plant of the family Apocynaceae is used to treat many of the fatal diseases (**Jaleel *et al.,* 2009**). There are about two common cultivars of *C. roseus* which are named on the basis of their flower color that is the pink flowered 'Rosea' and the white flowered 'Alba' (**Sain and Sharma, 2013**). *C. roseus* is extensively cultivated in northern India for its ever increasing demand in pharmaceutical and medical industry **(Patil and Ghosh, 2010; Nayak *et al*., 2006)**. *Catharanthus roseus* has been used in folk medicine to treat sore throat, mouth ulcer, diabetes, high blood pressure, muscle pain and cancer treatment (**Devi *et al*., 2013; Sain and Sharma, 2013**). Most pathogens are developing resistance against many of the currently available antimicrobial drugs (**Patil and Ghosh, 2010; Devi *et al.,* 2013; Ramya *et al*., 2008**). Resistance in pathogens has increased at high rate and multi drug resistant microorganisms have exacerbated the situation (**Nino *et al*., 2006; Schinor *et al*., 2007**). Emerging and re-emerging infections and microbial drug-resistance pose a challenge to the global public health. Plants provide unique elements which are indispensable for novel drug discovery (**Essawi and Srour, 2000; Goyal *et al.,* 2008; Khalil, 2012**). There is an urgent need to search and develop cheaper plant based drugs. Recent attention has been on compounds. The increased preference of herbal medicine has consequently propelled the search for pharmaceutical remedies against different ailments from plants (**Tugume *et al.,* 2016**). *C. albicans* cause infections that range from superficial infections of the skin to life-threatening systemic infections (**Mayer *et al*., 2013**). Multi-drug resistant diarrhoeagenic *E. coli* have been isolated from children (**Vila *et al.,* 1999**). According to **Mathekaga and Mayer (1998)** screening methods provide preliminary observations necessary to select crude plant extracts for further chemical and pharmacological investigations. Little research has been done on phytochemical and antimicrobial properties of *Catharanthus roseus* in Kenya. This study aimed at investigating the phytochemical and antimicrobial properties of leaf extracts of two cultivars of *Catharanthus roseus* on *Escherichia coli* and *Candida albicans*.

2. Material and methods

2.1 Field collection

Catharanthus roseus leaves of the two cultivars were collected around Maseno University Siriba campus, near Maseno Anglican Hospital. They were identified at the Maseno University Herbarium. The dirt adhered to the specimen was cleaned off by shaking the plant as well as washing using tap water then they were sterilized using methylated spirit. They were spread on the sterilized laboratory bench to air dry for three weeks at room temperature, away from direct sunlight.

2.2 Extraction

After collection and identification, the leaves were air-dried in the laboratory for two weeks and the dried leaf material were weighed using electronic weighing balance, and grinded with electric grinder into fine powder (**Balaabirami and Patharajan, 2012**). Fifty (50g) dry powder of each variety were macerated cold in200ml of 70% ethanol for one week in a maceration tank at room temperature. Filtration was done using the whatmann no. 1 filter papers and the filtrate further transferred to the rotary evaporator (manufactured by Tokyo Rikakinah Co. Ltd-Eyela of type SB-1000 and operates at AC 230V, 50Hz, 1.1KVA) where ethanol evaporated at 78^0C leaving semisolid substances which were left to solidify and dry. After drying, the masses of the dry extracts were determined and the extracts kept safe at room temperature until usage.

2.3 Culturing of microorganisms

The microorganisms used in the antimicrobial activity of *Catharanthus roseus* were *Escherichia coli* and *Candida albicans.* The microbes were obtained from Maseno University Botany Laboratory. They were cultured using the Nutrient Agar (N.A) for bacterium and Potato Dextrose Agar (PDA) for fungi according to **Chon and Nelson (2004).**Test cultures were prepared by transferring a loop full of *Escherichia coli* from stock culture nutrient broth and incubated at 37^0C for 24h. *Candida albicans* were transferred into freshly prepared dextrose agar plates and incubated at 25^0C.

2.4 Comparative antimicrobial screening

The dry extracts were dissolved in sterile distilled water, to prepare solutions of 2.50, 5.0, and 7.5 mg/mL of different concentration of each extract. Circular discs of 6mm diameter were cut from Whatman no. 1 filter paper in the laboratory using a paper punch. They were dipped in the known concentrations of the plant extracts and allowed to absorb the plant extracts according to **Musyimi *et al.* (2008)**. Approximately 1×10^5cells/ml suspension of the *C. albicans* and *Escherichia coli* were aseptically inoculated on PDA and on nutrient agar petri dishes respectively. Sterile paper discs were then soaked in the prepared extracts of *Catharanthus roseus* leaf extracts and were transferred to the inoculated agar media, 3 discs each petri dish. They were sufficiently spaced to prevent the resulting zones of clearing from overlapping. The petri dishes were incubated for 48hrs at 27ºC. A paper disc impregnated in sterile water was used as control experiment (**Balaabirami and Patharajan, 2012**). The zone of inhibition was determined using a transparent ruler and a caliper to the nearest millimeter from the lower surface of the Petri dishes.

2.5 Phytochemical analysis

Phytochemical screening was done according to **Trease and Evans (1983)** and **Harbourne (1973)**.

Test for alkaloids
Two grams of the extract were extracted by warming it for 2 minutes with 20ml of 1% H_2SO_4 acid in a 50ml conical flask on a water bath, with intermittent shaking. One drop of Meyer's reagent was added to 0.1ml supernatant in a semi-micro tube. A cream precipitate indicated the presence of alkaloids.

Test for flavonoids
5 milliliters of dilute ammonia solution were added to a portion of the aqueous filtrate of the extract followed by addition of concentrated H_2SO_4. A yellow colouration indicated the presence of flavonoids.

Test for tannin
About 0.5 g of the dried powdered samples was boiled in 20ml of water in a test tube and filtered through Whatman No. 42 filter paper. A few drops of 0.1% ferric chloride were added. A brownish green or a blue-black coloration indicated the presence of tannins.

Test for phenols
Ferric chloride test was carried out where the extract was diluted to 5ml with distilled water. Then, a few drops of neutral 5% Ferric chloride solution were added. A dark green or a blue-black colour indicated the presence of phenolic compounds.

Test for steroids
Two ml of acetic anhydride was added to 0.5 g ethanolic extract of each sample with 2ml H_2S0_4. Colour change from violet to blue or green indicated the presence of steroids.

Test for saponins
About 2 g of the powdered sample was boiled in 20ml of distilled water in a water bath and filtered. Ten millilitres of the filtrate were mixed with 5ml of distilled water and shaken vigorously to form a stable persistent froth. The froth was mixed with 3 drops of olive oil and shaken vigorously, and then was observed for the formation of emulsion.

Test for terpenoids
Five millilitres of each extract was mixed with 2ml of chloroform, and concentrated sulphuric acid was carefully added to form a layer. A reddish brown colouration that formed at the interface indicated the presence of terpenoids.

Test for Cardiac glycosides
Five ml of extract was treated with 2ml of glacial acetic acid containing a drop of $FeCl_3$ solution. This was then underplayed with 1ml conc. H_2SO_4. A brown ring of the interface indicated a deoxy-sugar characteristic of cardiac glycosides.

2.6 Data analysis

The data collected were subjected to analysis of variance (ANOVA) using SAS statistical package. The treatment means were separated and compared at ($p<0.05$).

3. Results

Phytochemical screening of leaf extracts of the two cultivars of *Catharanthus roseus* confirmed the presence of alkaloids, terpenoids, saponins, tannins, phenols and flavonoids in the ethanol leaf extracts, but lacked steroids and glycosides (Table 1).

Table 1 Phytochemical screening of secondary metabolites in the leaf extracts of two cultivars of *Catharanthus roseus*

Phytochemicals	Alba leaves	Rosea leaves
alkaloids	+	+
steroids	-	-
terpenoids	+	+
saponins	+	+
tannins	+	+
phenols	+	+
flavonoids	+	+
glycosides	-	-

+: Present, -: Absent

The two plant cultivars extracts were found to possess antimicrobial activities on *E. coli* and *C. albicans* (Table 2 and Table 3). The Alba cultivar leaf extracts showed higher inhibitory activity on the bacterial strain (*E. coli)* compared to the Rosea cultivar leaf extract. The leaf extract was found to be most active against *C. albicans* (inhibition zone of 9mm) compared to *E. coli* (inhibition zones of 8.99 mm).

Table 2 The growth inhibitory effect of plant leaf extract of *Catharanthus roseus* cultivars *on Escherichia coli* and *Candida albicans*

Catharanthus roseus Cultivars	*Microbe*	*Extract concentration (mg/mL)*	*Zone of inhibition (mm)*
Alba	*E. coli*	0.0	6.00±0.00
		2.5	8.00±0.51
		5.0	12.10±2.00
		7.5	16.89±1.48
Alba	*C. albicans*	0.0	6.00±0.00
		2.5	9.00±0.84
		5.0	9.33±0.84
		7.5	11.33±1.17
Rosea	*E. coli*	0.0	6.00±0.00
		2.5	6.11±0.40
		5.0	8.11±0.48
		7.5	8.78±0.78
Rosea	*C. albicans*	0.0	6.00±0.00
		2.5	9.00±0.77
		5.0	9.67±2.22
		7.5	11.67±1.35

Table 3 Comparative Antimicrobial effects of different concentrations of leaf extract of *Catharanthus roseus cultivars* on *Escherichia coli* and *Candida albicans*

Concentration of Extracts (mg/mL)	*Diameter of inhibition (mm)*
0	6.00d
2.5	8.19c
5.0	9.64b
7.5	12.17a
LSD	1.3802
Microbes	
E. coli	8.99a
C. albicans	9.00a
LSD	0.976
Cultivars	
Alba	9.8321a
Rosea	8.1667b
LSD	0.976

Means with the same letter down the column are not significantly different. Data presented are means of three replicates.

4. Discussion

Plants are important source of potentially useful structures for the development of new chemotherapeutic agents. Plants offer the local population with immediate and accessible therapeutic products (**Mwaura *et al.,* 2020; Bruck *et al.*, 2004**). Plants and their secondary metabolites have shown great potential as antibacterial and antifungal sources (**Bikash *et al.,* 2011**). In Kenya, traditional medicines play a major role in primary healthcare and upkeep of rural communities (**Kokwaro, 1988; Kisangau and Kokwaro, 2004**). Over 70% of the Kenyan population relies on traditional medicine as their primary source of healthcare (**Odera, 1997**). Phytochemical screenings help to reveal the chemical nature of plant constituents (**Enwuru *et al., 2008***). The results indicated that the two cultivars of *Catharanthus roseus* extracts contained alkaloids, terpenoids, saponins, tannins, flavonoids and some phenolic compounds. Steroids and cardiac glycosides were absent in the leaves. These results are in agreement with those of **Phani *et al.* (2013); Sheeraz *et al.* (2013)** and **Giri *et al.* (2012)**. Composition of secondary metabolites varies from species to species, climatic

conditions and the physiological state of developments of the endemic plants (**Musyimi *et al*., 2008; Hussain and Deeni, 1991**). The biochemical composition of plants is the most common parameter used for the characterization of plants (**Deepak *et al.,* 2009**).The presence of these bioactive compounds in ethanolic leaf extracts may account for the antimicrobial activity. The detected compounds in this study have been vastly reported for their antimicrobial activities (**Tiwari *et al*., 2011; Sheeraz *et al*., 2013)**. The curative properties of medicinal plants have been attributed to the presence of alkaloids, flavonoids, phenols, saponins, steroids in plants (**Britto and Sebastian, 2011**). Phenolic compounds are most abundant plant metabolites with a variety of antimicrobial properties (**Audu *et al*., 2007**). Alkaloids do not show direct antimicrobial actions but strengthens the immune system. If the immune system is too weak or the organism is too virulent then certain other medication, along with these compounds are given to show significant antimicrobial activity (**Patil and Ghosh, 2010**). Flavonoids inhibit many bacterial strains and important enzymes. Tannins have astringent properties which accelerate the healing of wounds and inflamed mucous membranes. Tannin compounds inhibit the growth of microorganisms (**Chung *et al.,* 1998**). Plants with tannins are used to treat non-specific diarrhea and inflammation of the mouth (**Shohel *et al.,* 2014**). The biological function of alkaloids, saponins and their derivatives are very important (**Stary, 1998**). Saponins dissolve in water to form foamy solutions and because of surface activity, some drugs containing saponins have a very high long history of usage. Preliminary screening tests may be useful in detecting the bioactive principles (**Doss *et al.,* 2009**). The antimicrobial activities of ethanol extract may be due to the presence of tannins, triterpenoids and flavonoids (**Mamtha *et al.,* 2004**). Differences in antimicrobial activity of medicinal plants are obviously related to differences in their contents of active compounds. These compounds having minimum side effect and can be easily substituted for antibiotics. Therefore, the presence of these phytochemicals could to some extent justify the observed antimicrobial activities in the current study. This may be due to structural differences between bacterial and fungal agents. Phytochemical compounds are responsible for antimicrobial activity against stomach pain and diarrhea pathogens (**Sahleand and Okbatinsae, 2017**). This study demonstrates that ethanol leaf extract of *Catharanthus roseus* are effective against *Candida albicans*. *Candida albicans* are very resistant fungi (**Khalil, 2012**). The results have shown that there was a significant difference between the plant cultivars. Alba cultivar was highly effective. However, these differences that were observed among the microbial activities could be due to the difference in the chemical composition of the plant extracts as revealed by phytochemical analysis. The differences could also be attributed to the differences in susceptibility of the test microorganisms to the phytochemicals and differences in cell wall structure and composition. In fact, it has been reported in other studies (**Tekwu *et al.,* 2012; Zavala *et al.,* 1999**). Plant extracts often show a higher activity against bacteria compared to fungi, and this may partly be due to differences in the cell wall synthesis and structure. The susceptibility of *E. coli* and *C. Albicans* to the ethanol extract is a clear indication that these plants can be further exploited as a potential source of antibacterial and antifungal compounds.

5. Conclusion

In this study ethanol leaf extracts of Alba and Rosea varieties of *Catharanthus roseus* inhibited the growth of *E. coli* and *C. albicans*. The findings indicate that ethanol leaf extract of *Catharanthus roseus* possess potential antibacterial and antifungal activity. This study has also revealed the presence of phytochemical constituents in the leaves except the steroids and cardiac glycosides in the *Catharanthus roseus.* The appreciable antimicrobial activities of ethanol extract noted in this study may be due to the presence of tannins, triterpenoids and flavonoids. The findings from this study support the traditional use of this plant. Moreover, the findings of this study add value to the traditional uses of these plants. In conclusion, the two plant cultivars leaves may be reliable sources of antimicrobials which can be used the development of novel drugs and the treatment of multi drug resistance pathogens*.* More research is needed to draw the comparison on effectiveness of various plant part extracts.

Declaration of interest

The authors declare that they have no conflicts of interest.

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Phytochemical, Nutritional and Pharmacological Potentialities of *Amaranthus spinosus* Linn. : A review

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Abstract

Amaranthus spinosus has long been cultivated in tropical and subtropical areas of the world, especially in South Asia. It is well accepted by the people for its nutritional, pharmacological, phytochemical, and therapeutic functions in the human body. Tender stems, leaves, shoots, grains and sometimes the whole part of *A. spinosus* are eaten by humans or fed to farm animals, which contain carbohydrates, proteins, fats, fibers, vitamins, minerals and many other phytochemicals. This review aims to represent the nutritional and pharmacological activities of *A. spinosus.* To have a better understanding, we have discussed the nutritional status of *A. spinosus*, its available phytochemicals and their functional properties. Further, we demonstrated the potentiality of *A. spinosus* in various disease condition by discussing its functional activities, which includes antioxidant, antidiabetic, immuno-modulatory, hematological, gastrointestinal, anti-inflammatory, diuretic, antimicrobial, antimalarial, anti-ulcer, antipyretic, and antigenic activity. The availability of various important phytochemicals along with their functional properties make *Amaranthus spinosus* valuable for pharmaceuticals and nutraceuticals industry.

Abbreviations

µg	Microgram
µM	Micromolar
A.	*Amaranthus*
ABTS	2,2´-azinobis-3-ethylbenzothiazoline-6-sulfonic acid
ASAE	*Amaranthus spinosus* aqueous extract
ASE	50% ethanolic extract of *Amaranthus spinosus*
BHA	Butylated hydroxyanisole
COX-2	Cyclooxygenase-2
DEX	Dexamethasone
DPPH	2,2-diphenyl-1-picrylhydrazyl
EC50	Median effective concentration required to induce a 50% effect
FST	Forced swimming test
GF1	GATA-binding protein 1
Hb	Hemoglobin
HCl	Hydrochloric acid
IC50	Half-maximal inhibitory concentration
IEF	Isoelectric focusing
kDa	Kilodalton
LD_{50}	Median lethal dose
MEAS	Methanolic extract of *Amaranthus spinosus*
mg	Milligram
mL	Milliliter
OECD	Organisation for Economic Co-operation and Development
PGE_2	Prostaglandin E_2
RBC	Red blood cells
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide electrophoresis
SS	Swimming stress
TST	Tail suspension test
WBC	White blood cells

1. Introduction

Plant-based medicine is potentially the oldest form of medicine, some of which are not only used as medicine but also as nutritional victuals that showed the value of in-depth scientific evaluation. *Amaranthus spinosus* Linn. (Family: Amaranthaceae) is a most frequent consumable vegetable which has many medicinal aspects and is commonly known as Katanote in Bengali, Pigweed in English, and Kanatabhajii in Hindi **(Alegbejo, 2014; Kawade *et al.*, 2013)**. It is widely distributed from tropical, subtropical, and some temperate regions **(Tanmoy *et al.*, 2014)**. The genus *Amaranthus* has approximately 60 species, but 20 are important as weeds and others are widely used as vegetables, cereals and ornamental plants **(Venskutonis & Kraujalis, 2013)**. Its tender stems, leaves, shoots and grains are eaten by humans which contain thousands of phytochemicals and prevents several chronic and degenerative disease. Phenolic compounds named quercetin, kaempferol glycosides and hydroxycinnamates are found in *A. spinosus* with the ranges from 305 mg/100 g to 329 mg/100 g **(Stintzing *et al.*, 2004)**. The stem of *A. spinosus* has anti-diabetic activity, and its effectiveness is proved for diabetes mellitus **(Preethi, 2013)**, the root is used for the treatment of malaria **(Bahekar & Kale, 2013)** and the whole plant contains α-spinasterols octacosanoate & saponin which are effective as anti-inflammatory substances **(Sengupta *et al.*, 2012)**. It is also proved that consuming *A. spinosus* extract significantly increases prolactin level and helps to produce breast milk in postpartum mothers **(Kuswaningrum *et al.*, 2017)**. It also contains a red

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pigment which is utilized as a coloring agent in foods or medicine **(Amabye, 2016)**.

This plant is largely utilized as vegetables because of its excellent source of antioxidant leaf pigments such as β-cyanin, β-xanthine, betalain and as a source of other pigments such as carotenoids, anthocyanin, chlorophylls. It is also is a great source of antioxidant phytochemicals such as β-carotene, vitamin C, phenolics and flavonoids **(Jiménez-Aguilar & Grusak, 2017; Sarker & Oba, 2019)**. In Malaysia, it is used as an expectorant as well as to enhance breathing in acute bronchitis **(Alegbejo, 2014)**. The decoction of this plant is used for toothache, the ash is used as salt, boiled leaves and roots are eaten as laxative, the whole plant is fed to livestock as forage, and it also has numerous pharmacological uses such as astringent, antidote to snake poison, menorrhagia, gonorrhea, internal bleeding, ulcerated mouths, nosebleeds, hepatic disorder, sudorific, eczema, galactagogue, febrifuge, diaphoretic, wounds, burns and dysentery **(Alegbejo, 2014; Rahman & Gulshana, 2014; Kawade *et al.*, 2013; Sarker & Oba, 2019)**. Along with all these potencies, *A. spinosus* also shows antimicrobial activity. This plant is widely found in roadsides, waste places and fields **(Tanmoy *et al.*, 2014)**.

To provide a descriptive overview of *A. spinosus,* nutritional and phytochemicals of this vegetables, evaluation methods, internal and external factors that influence its functional properties were summarized and reviewed.

2. Proximate composition

A. spinosus is an abundant source of dietary fibers. It also contains ash, moisture, crude fat and crude protein **(Sarker & Oba, 2018, 2019)**. After analyzing 100 g of its leaves, it was found the carbohydrate content was 1.16 g, energy 27 kcal, moisture 91 g, protein 4 g, fat 0.6 g, fiber 2.48 g, ash 2.76 g **(Figure 1A).** In dry weight per 100g, the mineral content was 38.4 mg iron (Fe), 968.7 mg Calcium (Ca), 912.4 mg magnesium (Mg), 816.3 mg phosphorus (P), 6.8 mg manganese (Mn), 1.2 mg copper (Cu), 6.8 mg zinc (Zn) **(Figure 1B) (Kawade *et al.*, 2013)**. It has high nutritive value because of its elevated concentration of antioxidant compounds, fibers, proteins and amino acids, predominantly lysine **(Tanmoy *et al.*, 2014)**.

3. Phytochemistry

There are abounding active phytoconstituents in *A. spinosus,* which belongs to the group of flavonoids, alkaloids, amino acids, lipids, glycosides, phenolics, steroids, terpenoids, saponins, betalains, catechuic tannins, betaines such as trigonelline and glycine betaine, and carotenoids. Amaranthosides, amaricins and a coumaroyl adenosine together with stigmasterol glycoside are also seemed to be present in this plant **(Ganjare & Raut, 2019)**.

Amaranthines, hydroxycinnamates, isoamaranthines, quercetins and kaempferols are mostly found in stems. Quercetin, α-xylofuranosyl uracil, β-D-ribofuranosyl adenine, β-sitosterol glucoside, 7-p-coumaroyl apigenin 5-O-β-D-glucopyranoside, amaranthoside, rutin, and amaricin are found in the whole plant. α-spinasterol and saponins are found in roots of the plant. Hectriacontane, oleanolic acid, D-glucose, D-glucuronic acid, aliphatic ester-α-spinasterol octacosanoate are generally found in leaves and less frequently in stems **(Tanmoy *et al.*, 2014)**. The main betalains in *A. spinosus* are found as quercetin, amaranthine, isoamaranthine, hydroxycinnamates and kaempferol glycosides. Coumaroyl flavone glycoside is identified from the *n*-butanol fraction of methanol extract. In the *A. spinosus* plant, the rutins and the quercetins are present as flavonoids. Rutin is identified in the whole plant powder. Separation and identification of amaranthoside, amaricin, and stigmasterol glycoside have become possible through the phytochemicals investigating method using *n*-butanol fraction of methanol extract of whole plant of *A. spinosus.* By using petroleum ether extract, α-spinasterol and hectriacontane are isolated. Some new compounds, such as aliphatic esters and saponins, specifically saponin I and saponin II were identified in *A. spinosus* **(Sarker & Oba, 2019; Tanmoy *et al.*, 2014)**.

The presence of all these compounds explicitly related to some medicinal properties of *A. spinosus*. Compounds like vitamin C, flavonoids and phenolics are phytochemical compounds which show most of the antioxidant activity in different plants including *A. spinosus.* It is evident that phenolic and flavonoid compounds may prevent or lessen the effect of particular cancer and cardiovascular diseases, along with the chronic and neurodegenerative types of diseases. *A. spinosus* has reported to provide antidiabetic, anti-inflammatory, anti-malarial, anti-fertility, anti-hyperlipidemic, spermatogenic and antimicrobial effects **(Figure 2) (Ganjare & Raut, 2019; Kawade *et al.*, 2013)**.

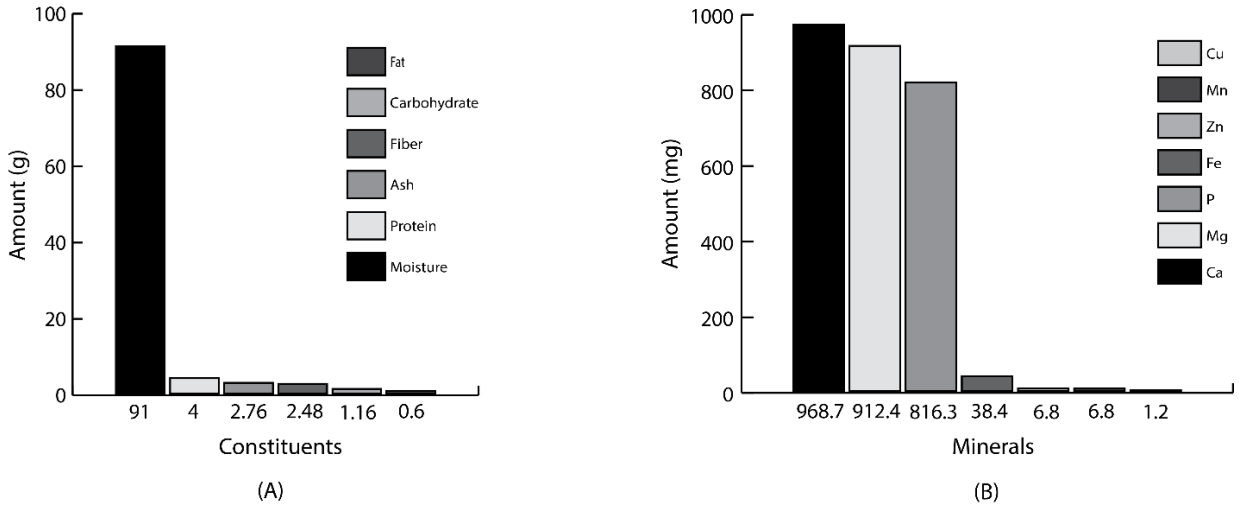

Figure 1 *A. spinosus* constituents and its mineral contents have been illustrated in the graphs, which are found in 100 gm weight. **(A)** Proximate composition or constituents of *A. spinosus* found in 100 gm of its leaves are demonstrated in this chart. This chart suggests that protein, ash, fiber and carbohydrates are quite good in *A. spinosus*, whereas fat content is the lowest in amount **(B)** Minerals found per 100 gm dry weight of *A. spinosus* has been demonstrated in the chart. It suggests that Ca, Mg and P are found in higher amount, where Fe, Zn, Mn and Cu are found in lower amount.

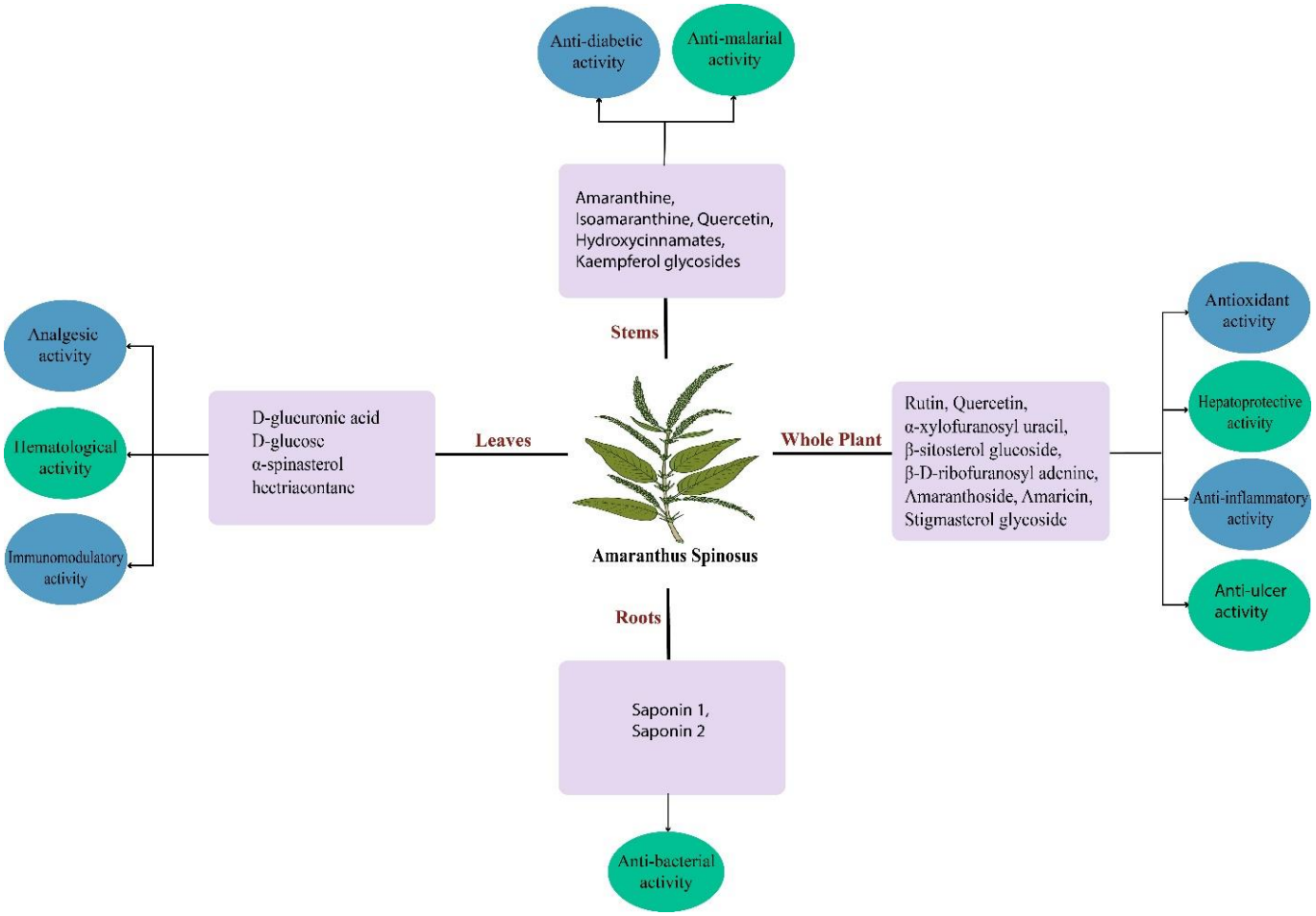

Figure 2 Phytochemicals found in whole plant, roots, leaves and stems of *Amaranthus spinosus* have been demonstrated in the rectangular boxes. Pharmacological activities of these phytochemicals have been indicated in the circles.

4. Phytochemical constituents

Most of the phytochemicals of *A. spinosus* are found in the matured leaves, because maximum metabolism in plants occurs in matured stage. To obtain the extract, leaves are air-dried at room temperature and allowed for grinding and finally extract is collected by solvent extraction. The analysis of *A. spinosus* to determine its phytochemical contents are done by standard procedures **(Amabye, 2016; Maiyo *et al.*, 2010)**. Numerous phytochemical and phenolic compounds are found in several extracts of *Amaranthus spinosus,* still some other phytochemical compounds did not show their presence in the extract. The wide range of phytochemical constituents, and their presence or absence in different extracts can easily be recognized if analyzed properly **(Table 1)**.

Amaranthus spinosus plant extract shows *in vitro* bioactivity because of the presence of innumerable phytochemical constituents **(Maiyo *et al.*, 2010)**. Moreover, biologically active phytochemical compounds largely contribute to the amelioration of public health **(Table 2)**.

Table 1 Quantitative screening for phytochemical constituents of *Amaranthus spinosus*

Constituents	Presence (+) or Absence (-)					Content	Reference
	Methanol	Ethanol	Hexane	Chloroform	Aqueous		
Alkaloids	+	-	-	-	-	13.14 ± 0.86	**(Amabye, 2016; Khanal *et al.*, 2015)**
Tannins	-	-	-	+	-	6.07 ± 0.93	**(Amabye, 2016; Maiyo *et al.*, 2010)**
Flavonoids	+	+	+	+	+	1.70 ± 0.56	**(Jiménez-Aguilar & Grusak, 2017; Khanal *et al.*, 2015)**
Saponins	+	+	-	-	-	53.0 ± 0.50	**(Amabye, 2016; Khanal *et al.*, 2015; Maiyo *et al.*, 2010)**

Table 2 Phytoconstituents and pharmacological activities of *Amaranthus spinosus*

Phytoconstituents	Plant part	Extract	Pharmacological activity	Reference
Rutin, Quercetin, 7-p-coumaroyl apigenin 4-O-β-D-glucopyranoside, α-xylofuranosyl uracil, β-sitosterol glucoside, β-D-ribofuranosyl adenine, Amaranthoside, Amaricin, Stigmasterol glycoside	Whole	Ethanol	Hepatoprotective activity	**(Azhar-ul-Haq *et al.*, 2004; Suryavanshi *et al.*, 2007; Zeashan *et al.*, 2008; Zeashan *et al.*, 2009b)**
	Whole	Petroleum ether, methanol, chloroform, aqueous	Antioxidant activity	**(Kumar *et al.*, 2010b; Kumar *et al.*, 2010c)**
	Whole	Ethanol	Anti-inflammatory activity	**(Olajide *et al.*, 2004; Sengupta *et al.*, 2012)**
	Whole	Aqueous	Anthelminthic activity	**(Baral *et al.*, 2010; Kumar *et al.*, 2010a)**
	Whole	Ethanol	Anti-diarrheal activity	**(Hussain *et al.*, 2009)**
	Whole	Ethanol	Anti-ulcer activity	**(Hussain *et al.*, 2009; Mitra, 2013)**
D-glucuronic acid and D-glucose, α-spinasterol, hectriacontane	Leaves	Ethanol	Analgesic activity	**(Banerji, 1973; Taiab *et al.*, 2011)**
	Leaves	Ethanol	Hematologic activity	**(Akinloye & Olorede, 2000; Olufemi *et al.*, 2003)**
	Leaves	Aqueous	Immunomodulatory activity	**(Alegbejo, 2014; B.-F. Lin *et al.*, 2005)**
	Leaves	Aqueous	Gastrointestinal activity	**(Kumar *et al.*, 2008)**
Amaranthine, Isoamaranthine, Quercetin, Hydroxycinnamates, Kaempferol glycosides	Stems	Methanol	Anti-diabetic activity	**(Sangameswaran & Jayakar, 2008; Stintzing *et al.*, 2004)**
	Stems	Aqueous	Anti-malarial activity	**(Hilou *et al.*, 2006)**
Saponins	Roots	Hexane	Anti-bacterial activity	**(Ahmad & Basha, 2006; Banerji, 1973, 1979, 1980; Vardhana, 2011)**
	Roots	Ethyl acetate	Anti-bacterial activity	

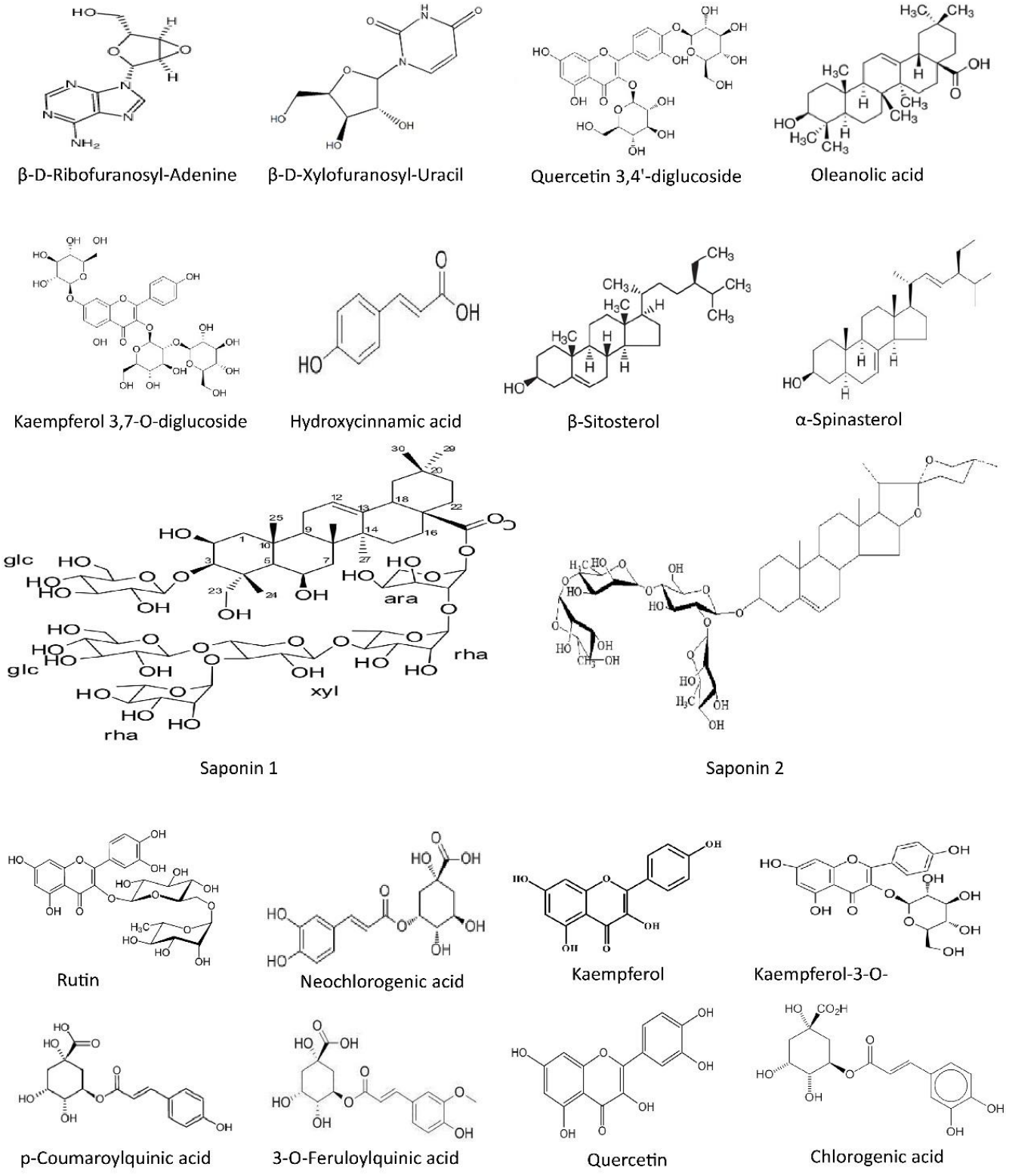

Figure 3 The chemical structures of some bioactive and phytochemical compounds found in different parts of *Amaranthus spinosus*

5. Nutritional and pharmacological activities

Several nutritional and pharmacological properties of *A. spinosus* are said to contain in traditional system. These have been scientifically substantiated by scientists which are discussed in the following sections.

The antioxidant activity

The antioxidant activity of *A. spinosus* is analyzed by non-enzymatic haemoglycosylation assay, which showed that secondary metabolites, named rutin and quercetin have inhibition tendency of haemoglycosylation up to 42% and 52% respectively **(Tanmoy *et al.*, 2014)**. Analysis of roadside *A. spinosus* reported they contain free radical scavenging system which can combat air pollution and it also contains Betalain pigment which shows strong antioxidant activity. The EC50 values range from 3.4-8.4 µM **(Jhade *et al.*, 2009)**. The

antioxidant activity is also examined by determining the oxidation of linoleic acid **(Amabye, 2016)**. Some of the methods include DPPH or 2,2-diphenyl-1-picrylhydrazyl scavenging; superoxide anion radical scavenging; nitric oxide scavenging; hydroxyl free radical scavenging; and ABTS or 2,2´-azinobis-3-ethyl-benzothiazoline-6-sulfonic acid radical scavenging assays **(Kumar *et al.*, 2010c)**.

Table 3 The antioxidant activity of *Amaranthus spinosus* illustrated by several methods

Evaluation method	Standard antioxidant	Extracts used	Conc. used	Preventive effect	Reference
Non-enzymatic haemoglycosylation assay	α-tocopherol (vitamin E)	Petroleum ether	0.5 mg/mL 1 mg/mL	13.1 % 16.4 %	**(Kumar *et al.*, 2010b)**
		Chloroform	0.5 mg/mL 1 mg/mL	5.7 % 12 %	
		Methanol	0.5 mg/mL 1 mg/mL	36.91 % 56.07 %	
		Aqueous	0.5 mg/mL 1 mg/mL	22.2 % 31.01 %	
DPPH radical scavenging assay	Ascorbic acid (vitamin C)	Methanol	50-250 µg/mL	87.50 ± 3.52 µg/mL IC_{50} value	**(Bulbul *et al.*, 2011)**
ABTS radical scavenging assay	Ascorbic acid (vitamin C)	Methanol	25-250 µg/mL	147.50 ± 2.61 µg/mL IC_{50} value	**(Kumar *et al.*, 2010c)**
Hydroxyl radical scavenging method	Butylated hydroxyanisole (BHA)	Ethanol (50%)	400 µg/mL	140-145 µg/mL IC_{50} value	**(Zeashan *et al.*, 2009b)**

The antidiabetic activity

A. spinosus has alpha amylase enzyme, which is a potential compound associated with carbohydrate digestion and glycemic balance. Antidiabetic activity was studied by introducing methanolic extract of *A. spinosus* stem in diabetic rats **(Jhade *et al.*, 2009)**. The antidiabetic potential of methanolic extract of *A. spinosus* was diagnosed through *in vitro* alpha amylase inhibition by a compound named CNPG-3 or 2-chloro-4-nitrophenyl-α-D-maltotrioside, in alloxan-induced diabetic rats. The study manifested significant restraining capacity of glucose level on a 15-day study. *A. spinosus* leaf extract also reduces hyperglycemia by abating pancreatic cells damage **(Figure 4)** and oxidative stress in streptozotocin-nicotinamide induced diabetes albino rats **(Mishra *et al.*, 2012; Tanmoy *et al.*, 2014)**.

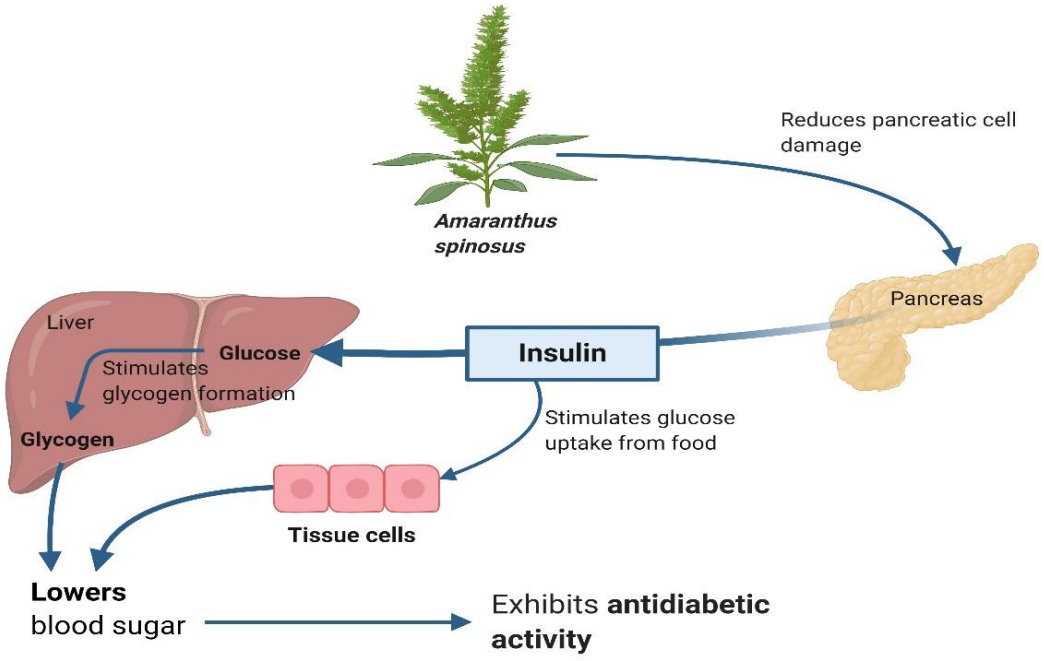

Figure 4 Active phytochemicals of *Amaranthus spinosus* can reduce pancreatic cell damages. Insulin released from pancreatic cells stimulate both glucose uptake and glycogen formation, therefore, manifests antidiabetic activity by lowering the blood sugar level.

The immuno-modulatory activities

A. spinosus has been demonstrated to possess immunological effects. It was determined by investigating the stimulatory effects of aqueous *A. spinosus* extract on spleen cells of female albino rats. The result showed that the extracts affiliate in stimulation of more B lymphocytes without increasing T lymphocytes level, and the immuno-stimulating effects of aqueous extract help B lymphocyte activation and proliferation of T-cell *in vitro.* Several studies substantiated that the extract of this plant can significantly escalate the splenocyte growth. The results also proved immuno-modulatory activity via direct *in vitro* B lymphocyte activation*.* A new immuno-stimulatory protein (GF1) having molecular mass of 313 kDa, which is thought to be a glycoprotein and heat labile, have 309 times higher immunostimulatory activity than that of water extract. This compound is highly potential for immune pharmacological use **(Ganjare & Raut, 2019; Gotyal *et al*., 2016)**.
Immuno-modulatory effects were also discovered using dexamethasone (DEX)-induced apoptosis in murine primary splenocytes and wild *A. spinosus* water extract. The result determined that *A. spinosus* water extract is capable of inhibiting the spontaneous, as well as the DEX induced apoptosis of splenocyte cells **(Lin *et al*., 2008)**. Cell-mediated immune response by delayed type of hypersensitivity reaction to sheep RBC, and humoral immune response measured by hemagglutination antibody tire also corroborated the immuno-modulatory activity. The result showed both immuno-modulatory and immunosuppressant activity due to presence of various glycosides, steroids and other phytochemicals. Aqueous and alcoholic extracts showed more immuno-modulatory effect, and petroleum ether extract showed immunosuppressant effect **(Tatiya *et al*., 2007)**.

The hematological activity

There has been a study on the effect of aqueous extract of leaves of *A. spinosus* on hematological parameters along with blood coagulation time in rat model, which showed little changes in the hematological activity and several enzymes level such as glutamate pyruvate transaminase, alkaline phosphatase and serum glutamate oxaloacetate transaminase **(Akinloye & Olorede, 2000)**.
In another study, *A. spinosus* leaf extract was fed to growing pigs and administered to determine its effects on packed cell volume, white blood cell, red blood cell, and hemoglobin concentration. The result showed that there has been temporary decrease in levels of packed cell volumes, white blood cell, red blood cell and hemoglobin **(Olufemi *et al*., 2003)**.
Change in hematocellular constituents of albino rats using *A. spinosus* methanolic extract has been also been diagnosed, which showed that WBC, RBC and Hb level was highly restored after using *A. spinosus* methanolic extract **(Gul *et al*., 2011)**.

The hepatoprotective activity

A. spinosus extract (whole) in 50% ethanol can be assessed against carbon tetrachloride induced hepatic damaged rats. The *A. spinosus* whole plant in 50% ethanol extract was fed to the carbon tetrachloride induced rats for 14 days and introduced oral doses of 100, 200 and 400 mg/kg in an experiment. The result showed substantive escalation of different enzymatic levels, such as serum glutamate pyruvate transaminase, glutamate oxaloacetate transaminase, and serum alkaline phosphatase; also, the bilirubin level came back to normal significantly. Conclusively, hepatoprotective activity is significant at higher dose of *A. spinosus* ethanol (50%) extract against CCl_4 inducing hepato-toxicity, because 400 mg/kg dose showed greater hepatoprotective activity than other doses **(Zeashan *et al*., 2008)**.
In another study, 50% ethanolic extract of *A. spinosus* whole plant is evaluated for hepatoprotective and *in vitro* antioxidant activity. Standard gallic acid curve (0-1.0 mg/mL) and an antioxidant naming butylated hydroxy anisole showed both the total phenolic compounds level and the hepatotoxicity reduction capacity of ASE respectively. The result had shown the hepatotoxicity reducing capability of ASE is 2.26 times of BHA and total polyphenolics expressed as gallic acid equivalent was 336 ± 14.3 mg/g, which proved that ASE possesses significant hepatoprotective activity **(Zeashan *et al*., 2009b)**.

The gastrointestinal activity

To determine the effect of *A. spinosus* in gastrointestinal tract, aqueous extract of *A. spinosus* was evaluated in mice using charcoal meal method. In the experiment, first group served as the basis of control, second group was made the standard, and the remaining 3-5 groups were fed *A. spinosus* aqueous extract at a dose of 50, 100 and 200 mg/kg weight of the mice respectively. Result of the experiment showed significant gastrointestinal motility at 100 mg/kg dose **(Kumar *et al*., 2008)**.
Another *in-vivo* experiment in mice using crude extract of *A. spinosus* showed the laxative activity, which is partially intervened through the cholinergic action **(Chaudhary *et al*., 2012)**.

The anti-inflammatory activity

The anti-inflammatory activity of *A. spinosus* has been evaluated by an experiment in which the methanolic extract of *A. spinosus* was evaluated in various animal models. The extract inhibited the carrageenan inducing mice paw edema at a dose of 25-100 mg/kg and helped in inhibition of acetic acid inducing increased vascular permeability. Though the 50 and 100 mg/kg extract dose produced gastric erosion in rats along with indomethacin, 25 mg/kg extract along with tween-80 (polysorbate-80) did not show this effect in macroscopic evaluation. However, several experiments corroborated the anti-inflammatory properties of *A. spinosus* **(Baral *et al*., 2010; Olajide *et al*., 2004)**.

The diuretic activity

To examine the diuretic activity of *A. spinosus* aqueous extract (ASAE) in rats, various doses of ASAE (200, 500, 1000, 1500 mg/kg), thiazide (10 mg/kg) and vehicle were fed to rats orally and their urine was collected and analyzed after 24 hours. The result showed that ASAE increased the concentrations of Na^+, K^+, Cl^- and caused alkalization of urine. The report of this test proved that the *A. spinosus* may act as a thiazide-like diuretic, occupying the carbonic anhydrase inhibiting property **(Amuthan *et al*., 2012)**.

The antidepressant activity

By using Forced swimming test and Tail suspension test patterns, the anti-depressant activity of the methanolic extract of *A. spinosus* (MEAS) was examined, where reference standards were Escitalopram and Imipramine. The result of this experiment showed that the MEAS cause significant diminution of immobility in FST and TST comparing with Escitalopram and Imipramine **(Kumar *et al*., 2014)**.

The Antimicrobial Activity

The *A. spinosus* has various pharmacologically active compounds, which shows antimicrobial activities in the disc

diffusion essay. Even though the trend for anti-fungal activity is same as of the anti-bacterial activity, the potency towards fungal strain is not as effective as for bacterial strains **(Amabye, 2016)**. Terpenoids show antimicrobial activity against bacterium, fungus, virus and protozoa, and it is used to control *Listeria monocytogenes.* These terpenoid compounds demonstrate antimicrobial activity by lipophilic membrane disruption mechanism. If the hydrophilic behavior of ent-kaurene diterpenoid compounds can be increased by addition of methyl group, this will reduce the antimicrobial activity of these compounds. Flavonoids generally are known to show response against microbial infections. Flavonoids form complexes with extra-cellular and soluble proteins, as well as with bacterial cells *in vivo.* This is why flavonoids are also effective as antimicrobial agents **(Maiyo *et al*., 2010)**.

Usage of dichloromethane extract of *A. spinosus* moderately shows antiprotozoal activity, especially on *Blastocystis hominis* when 2 mg/mL dose is introduced **(Kawade *et al*., 2013)**. Ethanol extracts of *A. spinosus* leaves show anthelminthic activity if fed to growing pigs. The result shows that mean egg count of helminths, predominantly *Ascaris suum,* is remarkably decreased due to ethanol extract of *A. spinosus* leaves **(Assiak *et al*., 2002)**.

Table 4 Antimicrobial activities of *A. spinosus* methanol leaf extract

Organism types	Test Organisms	Extract	Zone of growth inhibition (mm)	Reference
Bacteria	*Staphylococcus aureus*	Methanol 100%	24.0	**(Amabye, 2016)**
Bacteria	*Escherichia coli*	Methanol	11.6 ± 0.2	**(Barku *et al*., 2013)**
Bacteria	*Bacillus subtilis*	Methanol	8.0 ± 0.26	**(Cherian & Sheela, 2016)**
Bacteria	*Salmonella typhi*	Hexane 100%	13.0 ± 1.2	**(Maiyo *et al*., 2010)**
Bacteria	*Klebsiella* sp.	Distilled water	7.0	**(Sheeba *et al*., 2012)**
Fungi	*Fussarium solani*	Methanol 100%	17.0	**(Amabye, 2016)**
Fungi	*Aspergillus flavus*	Methanol	6 ± 0.76	**(Cherian & Sheela, 2016)**

The antimalarial activity

Screening of *A. spinosus* shows impressive antimalarial activity in mice when evaluated by suppressive antimalarial assay for 4 days. A study on *Plasmodium berghei berghei* parasite strain, which was inoculated in mice, had been inhibited greatly by *A. spinosus* at elevated doses of the extract produced from the plant. With 100 mg/kg dose introduction, the inhibition percentage of parasitemia is low, but increased doses (300 mg/kg to 900 mg/kg) had given better inhibition percentage. Though the plant has lower antimalarial activity compared to malarial medicine chloroquine, it's parasitemia inhibition percentage can also be increased by introducing relatively higher doses (1000 mg/kg or more) **(Hilou *et al*., 2006)**.

The anti-ulcer activity

In an interesting study on antiulcerogenic activity of *A. spinosus,* it was found out that *A. spinosus* possesses anti peptic ulcer activity when powdered leaves of *A. spinosus is* fed to gastric and duodenal ulcerated albino rats. The result shows that *A. spinosus* leaves can immensely protect ethanol inducing peptic or gastric ulcers, and cysteamine inducing gastric ulcers (duodenal ulcers). Though the anti-ulcer activity of *A. spinosus* showed less effect than a peptic ulcer drug, omeprazole, still it's role is crucial because of no known side effects or prolonged effects **(Debiprasad *et al*., 2013)**.

In another study of anti-ulcer activity, 50% ethanolic extract of *A. spinosus* (ASE) whole plant was evaluated in rats. Induction of acute gastric mucosal lesions were monitored using three different assay models in the investigation. The result proved that ASE can significantly protect both ethanol induced and aspirin induced ulcer **(Hussain *et al*., 2009)**. One of the important factors in ulcerogenesis is lipid peroxidation, and studies have substantiated that ASE can control this type of lipid peroxidation **(Hussain *et al*., 2009; Sairam *et al*., 2002)**.

Search for new drugs of gastric ulcer led to another experiment on *A. spinosus* for its antiulcer activity. Result of the study showed that leaves of *A. spinosus* can prevent the loss of gastric protein, and the lipid peroxidation due to aspirin induced ulceration. Simultaneously, the leaves of this plant had increased gastric mucin, and showed cytoprotective effect against gastritis **(Mitra *et al*., 2014)**.

More studies showed that roots and stems along with leaves of *A. spinosus* exhibits antiulcer activity in albino rats. The effect is prevalent against ethanol, HCl, Swimming stress (SS), pyloric ligation, and indomethacin inducing peptic or gastric ulcer. However, the result showed that utilization of powdered roots of *A. spinosus* with one of the ulcerogenic medicine can bring about more ulcerative protection in ethanol, HCl, SS, pyloric ligation, and indomethacin induced gastric ulcer than utilization of stems and leaves of *A. spinosus* **(Mitra, 2013)**.

The antipyretic and antinociceptive activity

Antipyretic drugs can suppress the expression of COX-2 so that the higher temperature of the body is reduced through inhibition of PGE_2 biosynthesis, whereas natural COX-2 inhibitors have fewer side effects **(Luo *et al*., 2005)**. Methanol extract of *A. spinosus* shows antipyretic activity without inhibition of COX-2 expression when introduced to rats with yeast-induced elevated level of body temperature **(Lakshman & Jayaveera, 2011)**. The antipyretic effect of *A. spinosus* is quite significant, and is similar to the paracetamol group when the introduced dose is 400 mg/kg **(Bagepalli *et al*., 2011; Kumar *et al*., 2010c)**. In a study, 50% ethanolic extract of *A. spinosus* (ASE) had shown antinociceptive activity when introduced to male swiss albino rats by some methods, including acetic acid test, formalin test, tail suspension test and hot plate test. Result from the study had shown that ASE possesses the central as well as the peripheral antinociceptive activity, and significantly blocks pain in the first phase at higher dose of the extract (400 mg/kg). In the second

phase, ASE had blocked pain from inflammation with introduction of all the doses **(Zeashan *et al*., 2009a)**.

The antigenic and allergenic activity

Pollen antigen standardization can assist in immunotherapy and diagnosis of allergenicity. *A. spinosus* is an essential aero-allergen and it has significance in type 1 hypersensitivity disorders. SDS-PAGE and IEF Analysis of five *A. spinosus* pollen samples showed that seven protein fractions have IgE binding capacities and nine proteins have allergenic properties **(Singh & Dahiya, 2002)**.

6. Toxicities

The toxicity of *A. spinosus* was detected by scientists using OECD guidelines. By following this guideline, lethal dose 50% (LD50) was determined in a lab experiment using albino rats. The dose was 5 mg/kg, 50 mg/kg, 200 mg/kg, 300 mg/kg (later the dose was 250 mg/kg and 300 mg/kg for further study) with sufficient amount of water orally in a single dose. The result showed *A. spinosus* extract did not cause any toxicity in the animals up to 2000 mg/kg **(Jhade *et al*., 2011; Mishra *et al*., 2012)**.
The genotoxicity of *A. spinosus* leaf extract was determined using meristematic root cells of *Allium cepa* and the antigenotoxic effects was determined by evaluating against H_2O_2-induced genetic damage in *Allium cepa.* The repot of this experiment manifested that higher dose of *A. spinosus* extract cause mitodepressive and clastrogenic effects, and lower dose of the *A. spinosus* extract reverts the clastrogenicity caused by H_2O_2. This clastrogenicity, which is induced by the *A. spinosus* extract demonstrates its genotoxicity. These results determined that *A. spinosus* extract has genotoxic as well as the antigenotoxic property **(Prajitha & Thoppil, 2016)**.

7. Conclusion

A. spinosus is abundant in phytochemicals. This review of *A. spinosus* shows that it holds nutritional potential along with various pharmacological properties. Leaf extract of this plant shows antimicrobial activities, especially against food-borne pathogens, and some fungus. Though some detailed studies have been done on this plant, it has not been developed as a drug yet. More exhaustive data on *A. spinosus* is not available till now even though the study on this potential plant was started in 1970s through analyzing its grain composition. Data on extraction of super-critical fluid and fractionation, high pressure and extrusion processing techniques are unavailable. Yet the screening or analysis within last 3 decades revealed so many information of this plant, such as its application for high quality baked goods production, edible films, functional ingredients etc. More extensive research and analysis is required for the cataloging, documentation and commercialization of this plant, considering its potentialities.

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Declaration of interest

All authors declare that there is no conflict of interest.

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Participation of Microorganisms in Milk and Milk-products Contamination and Safety

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Abstract

Milk and milk-products represent the main basic nutritional healthy food in the human diet; however, milk is also a favorable source of microbial infection for human health when milk and milk products are consumed without applying hygiene milk practices methods such as pasteurization and other effective methods to avoid contamination risk. The presence of microorganisms in milk could result in spoilage and severe diseases to humans. Several recent preservation systems such as heating, refrigeration, and the addition of safe antimicrobial compounds can be used to reduce the risk of outbreaks of dairy product poisoning. Proper food control programs must be implemented in all countries around the world to ensure the safety of food and dairy products. Investigators reported the importance of applying effective hygiene practices during milking and handling of raw milk to reduce the risk of contamination on the farm and in the milk processing plant in the industry.

1. Introduction

The control and disposal of contaminated and undesirable microorganisms in dairy and veterinary industries are very important in determining the quality of their final products **(Pal, 2014).** In dairy products some beneficial species of microorganisms are required for the production process of many dairy products such as Yogurt and cheese making **(Settanni and Moschetti, 2010; Singh *et al.,* 2016).** These include the conversion of milk constituents by enzymes of various species to attain the desirable flavor, taste, aroma, ripening and texture of these dairy products **(McSweeney *et al.,* 1997Ayad *et al.,* 2001)**. Buttermilk and Yogurt are examples of fermented milk products depending mainly on characteristic microflora that is responsible for their flavor and texture **(Ali *et al.,* 1995).** On the other hand microbes are undesirable in milk and their products causing disease. To ensure the safety of dairy products to consumers scientists should checked for decolorization, rancidity, ropiness, putrefaction, gassiness and many other defects that caused by different harmful microorganisms **(*Garcha, 2018).*** In this connection, many hygienic milk practices such as pasteurization, storage, handling, transport and distribution before consumption have greatly decreased the threat of milk-born diseases **(Yogesh *et al.,* 2012)**. Examples of the main discovered bacterial pathogens in milk and milk products are *Escherichia coli, Salmonella sp., Bacillus cereus, Campylobacter jejeni, Yersinia enterocolitica* **(Lubote *et al.,* 2014).** Some other genera of filamentous fungi producing mycotoxins are able to grow on milk and milk products (such as *Penicillium, Aspergillus and Fusarium*) that can be a fatal hazard to the consumers. Although some molds are responsible for ripening of many types of cheese (such as Roquefort and Camembert) and their enzymes such as amylase in making bread or citric acid used in soft drinks, some of them are mainly responsible for food spoilage at room temperature up to 30^0C and low pH, and have minimum moisture requirement. Yeasts capable to ferment sugars to ethanol and CO_2 such as *Saccharomyces cerevisiae* (or Backers' yeast) and *Sach. carlsbergensis* are used mainly in the process of making bread and fermentation of most beers respectively and the fungus *Agaricus bisporus* is one of the most used mushroom as a food source. Psychotrophs are mainly involved in milk spoilage and mainly destroyed by pasteurization, however many bacterial species such as *Pseudomonas fragi* and *Ps. flurescens* have the ability to produce heat resistant extracellular proteolytic and lipolytic enzymes capable of causing spoilage (Table 1). This review sheds light on some microbes found in milk and milk products and the appropriate methods to get rid of them using many recent effective methods.

2. Microbial contamination of milk and Milk products

The microbial contamination of milk and milk products takes places usually during processing, storage, transport and distribution before consumption. Dairy-borne infections have been identified as an important economic problem and public health in all countries around the world **(Pal et al., 2014).** For this reason microbial dairy safety represents a significant global issue for the consumer and industry. Microbial contamination is one the leading causes of milk and milk-products spoilage. Spoilage of milk and milk-products involve any change, which renders them unacceptable for human consumption. This is

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mainly due to the presence of highly nutritious components in milk and dairy products make them especially good media for the growth of microorganisms **(Ledenbach and Marshall, 2009).** The infectious undesired microorganisms present in contaminated milk and milk-products include for example *Escherichia coli*, *Mycobacterium bovis*, *Mycobacterium tuberculosis, Yersinia enterocolitica*, *Salmonella* and *Listeria*. These microbes can cause serious disease for immunity compromised individuals, children and pregnant women **(Pal, 2007; FAO, 2013)**. It is worthy to mention that the process of pasteurization cannot destroy all pathogenic microorganisms in milk, as many investigators reported the presence of *E. coli*, *Yersinia enterocolitica, Salmonella* spp.and *Staphylococcus aureus*, **(Pal *et al*., 2012)**. In order to maintain the safety of milk and milk-products molecular, immunological and microbiological techniques should be implemented to detect the presence of these pathogens. The main cause of failure of processing and packaging systems is the development of resistant bacterial biofilms to chemical sanitizers on equipment surfaces **(Ledenbach and Marshall, 2009; Pal *et al*., 2013). Sarkar (2015)**, reported that the poor quality of raw milk is due to microbial contamination, improper temperature control and inadequate packaging system **(Sarkar, 2015).** Fluid dairy products get easily contaminated with microorganisms than the dried products such as *Streptoccocus, Staphylococcus, Pseudomonas* spp. **(Fernandes, 2008).**

3. Cheese

Cheese is a dairy product rich in protein, calcium, phosphorus and vitamins produced by casein coagulation and entrapment of milk in the coagulum **(Fernandes, 2008)**. Different sources including environment, handling and packaging are responsible for microbial contamination of cheese **(Pal *et al*., 2014)**. **Vrdoljak *et al*., (2016)** reported that *Listeria monocytogenes* is the most food-borne pathogen in cheese in the processing phase. Other investigators indicated that certain strains such as *Streptococcus thermophilus* and *Lactobacillus helveticus* are capable of producing carbon dioxide gas resulting to the presence of cracks in cheeses **(Ledenbach and Marshall, 2009).** Although cheese is considered as a safe food due to the presence of antagonistic properties of lactic acid bacteria in the process of cheese making **(Kousta *et al.,* 2010),** however food borne outbreaks were found to be a result of contamination with *Staphylococcus aureus* the main cause of mastitis in cows (Rabello *et al.,* 2007). *S. aureus infections in cheese is due to* improper handling or to the use of unpasteurized milk because this bacterium produces heat-resistant enterotoxin **(Delbes *et al.,* 2006; CDC, 2010)**. **Ryser, (2001)** reported that all various *Salmonella* serotypes have been involved in cheese-borne outbreaks and all *Salmonella* strains are gastroenteritis-inducing pathogens. In this connection, microbial risk assessment is the proper scientific method for preventing, regulating and understanding the risk caused by hazardous microorganisms in cheese **(EPA, 2012).**

4. Yogurt

Yogurt is a unique fermented type of dairy product containing many nutritional components including protein, vitamins, calcium, phosphorus and magnesium **(Pal *et al.,* 2015).** The addition of fruits and flavor compounds to yogurt improve required conditions for the growth of molds and yeasts, but after a while they die out due to the acidic medium and the antagonistic effect exerted by lactic acid bacteria. The composition of the pleasant flavor of mature yogurt is mainly due to the presence of about equal proportions of starter cultures namely *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, the former adds flavor and aroma to yogurt, however the latter is mainly responsible for acid production **(Yamani and Ibrahim, 2007; Goel *et al.,* 1971)**. Yogurts have been found to be contaminated with both spoilage and pathogenic microorganisms due to unhygienic production processes which give the yogurt unsatisfactory sensory quality **(Mbaeyi-Nwaoha and Egbuche, 2012; Makwin *et al.,* 2014)**. Pathogenic bacteria that can cause spoilage of yogurt include Gram-negative psychrotrophs and coliforms **(Willey *et al.,* 2008; Oyeleke, 2009; Yabaya and Idris, 2012)**.

5. Protection of dairy products from spoilage microorganisms

To give dairy products the desirable shelf-life it is of importance to implement protection from spoilage during their preparation, storage and distribution. Several preservation systems such as heating, refrigeration and addition of antimicrobial compounds can be used to reduce the risk of outbreaks of dairy products poisoning. Nowadays, the food industry investigates more modern preservation techniques to replace the traditional food preservation including milk products in order to be accepted by consumer demand for nutritious, tasty, natural and easy-to-handle food products. In this connection, sorbic acid, lactic acid, benzoic acid and acetic acid are the most common classical preservative agents that inhibit the growth of bacterial and fungal cells **(Arneborg *et al.,* 2000).** Sorbic acid was found to inhibit the outgrowth of bacterial spores. The process of freezing prevents microbial growth and their enzymes, therefore the ingredients should be added prior freezing to ensure safety of food-products **(Rawat, 2015)**. The microbiological standard methods used for indicator microorganisms as a predictor of the safety and quality of milk and dairy products differ from country to country, each have their specific tests, regulations or guidelines for the contaminated microorganisms. The most used methods include Standard plate count (SPC) [100,000/ml max individual bulk tank]; Somatic cell count (SCC) [750,000/ml max individual bulk tank]; Aerobic plate count (APC) [100,000/ml max.]; Test for Coliforms, *E.coli* and Psychrotrophs.

6. Microorganisms in dairy products

6.1 Psychrotrophic microbes

This type of microorganisms prefers to grow on cold temperature especially in raw milk at 3-7^{0}C. They can easily hydrolyze proteins and lipids for their growth. The proper salt concentration in the cottage cheese content insufficient to limit the growth of these contaminating bacteria. For this reason psychrotrophs are the bacteria that normally limit the shelf life of cottage cheese **(Ledenbach and Marshall, 2009).** Investigators reported that the presence and subsequent replication of populations of psychrotrophs may lead to the spoilage of milk **(Pinto *et al.*, 2006; Nörnberg *et al.*, 2010).** Development of molecular biology for bacterial identification has revealed the presence of psychrotrophic bacteria not previously detected by the traditional methods **(Raats *et al.*, 2011; Almeida and Araujo, 2013). Mcphee and Griffiths, (2011)** reported that the reduction in cheese yield mainly due to the enzymes secreted by psychrotrophs that affect rennet coagulation times and altered starter activity and growth rate. The main cause of cheese reduction is mainly due to the loss of soluble casein degradation products into the whey instead of forming a part of the curd **(Mcphee and Griffiths, 2011; Mankai *et al.*, 2012)**. **Beales, (2004)** reported that refrigeration of milk and dairy products alone or in combination with other methods including the addition of preservatives is the most proper means of preservation. Generally the

psychrotrophic bacteria in milk and milk products represented predominantly by Gram -ve genera including *Pseudomonas*, *Achromobacter*, *Serratia*, *Alcaligenes, Aeromonas*, *Chromobacterium* and *Flavobacterium* spp. and in much lower numbers by Gram +ve genera including *Streptococcus, Lactobacillus*, *Clostridium*, *Corynebacterium*, and *Microbacterium* spp **(Sørhaug and Stepaniak, 1997; Mcphee and Griffiths, 2011)**.

6.2 Coliforms

These microorganisms are Gram negative, facultative anaerobic, rod shaped bacteria capable of fermenting lactose to produce gas and acid, belong to the family *Enterobacteriacea* such as *E.coli*, *klebsiella, Enterobacter aerogenes*, **(Pal and Mahendra, 2015)**. The slow lactic acid production by starter cultures favors the growth and production of gas by coliform bacteria. In case of soft ripened cheeses production, the increase in pH during the process of ripening reflects directly to the increase of coliform bacterial growth **(Ledenbach and Marshall, 2009)**. Application of strictest sanitary measures during milking process in the farm and milk storage and transportation to the dairy industry is the best way to prevent coliforms contamination. Recently the major challenge for dairy producers is to prevent post-pasteurization contamination (PPC) with spoilage microorganisms including coliforms **(Ranieri and Boor, 2009; Martin *et al.,* 2011).** The detection of coliforms in dairy products and pasteurized milk play a major role as a hygiene indicator tool for contamination. Their growth at refrigerated storage temperatures are of concern for dairy industry which can result in degradation of the product in addition to unacceptable sensory characteristics due to the formation of proteolytic and lipolytic enzymes **(Nörnberg *et al.,* 2010).**

6.3 Listeria monocytogenes

L. monocytogenes is a Gram-positive, rod-shaped, non-spore-forming, and facultative anaerobe bacterium causing public health problems. Both normal and diseased animals are the main source of the food-borne human pathogen *Listeria monocytogenes* in milk and dairy products. The pasteurization of milk does not *eliminate the milk and dairy products contamination by this bacterium* **(Sukhadeo and Trinad, 2009; Gould *et al.,* 2013)**. Investigators described non-thermal technologies high hydrostatic pressure (HHP) and pulsed electric fields (PEFs) as new preservation methods to control and prevent the growth of food-borne pathogens including *L. monocytogenes* **(Norton and Sun, 2008; Tomasula *et al.,* 2014).** These methods are mainly used to avoid undesirable changes in the nutritional bioactive compounds such as vitamins, and pigments in addition to sensory properties such as texture, taste, and flavor and consequently reducing their acceptability by consumers **(Cebrian *et al.,* 2016; Barba *et al.,* 2017)**.

6.4 Spore-Forming Bacteria

The most resistant life forms known in milk and milk-products are the pathogenic and spoilage associated species belonging to Bacilli and Clostridia classes. Clostridium species are well known contaminants in milk due to their ubiquitous nature and can enter the milk chain from different sources and their biofilms are highly resistant to heat and disinfectants. Many species such as *Clostridium botulinum* and *Clostridium perfringens* produce toxins causing dairy products poisoning. In this connection many cephalosporins antibiotics such as cephalexin and the cephamycin cefoxitin, have been found to inhibit effectively sporulation **(Miyamoto *et al.,* 1997; Hao and Kendrick 1998; Doyle *et al.,* 2015; Gopal *et al.,* 2015; Kumari and Sarker, 2016).** Bacillus species such as *Bacillus licheniformis* and *Bacillus pumilus* have been reported as the most commonly identified species in raw milk **(Miller *et al.,* 2015).**

6.5 Fungi

Fungi have a diverse secondary metabolism producing a number of toxic and carcinogenic mycotoxins. Some spoilage molds are toxigenic while others are not **(Pitt and Hocking, 1997).** They grow at a pH range of 3 to 8 and attack a wide variety of foods including milk products, their spores can tolerate unsuitable environmental conditions but most of them are sensitive to heat treatment. Different fungal species have different optimal growth temperature; however some few others can grow on cold conditions. Spoilage fungi can be categorized into the following groups: a) Zygomycetes: These fungi have the ability to grow on simple carbon sources and require high water activity for growth. Examples of this group are *Rhizopus* and *Mucor* species. b) *Penicillium*: They are distinguished than other spoilage microorganisms by their reproductive structures that produce chains of conidia. They are able to produce antibiotics and other dairy products such as blue cheese. Some species of this genus can produce mycotoxins and others can attack refrigerated milk and milk products. In this connection, a related genus namely *Byssochlamys* is the most serious causing spoilage fungi due to its high heat resistance of its spores. c) *Aspergillus*: These fungi are generally resistant to high degree of temperature and low water activity as previously described in case of Penicillium genus. They prefer warmer climate for growth. Many of them produce ochratoxin, aflatoxins and mycotoxins and can affect many food sources such as grains, peanuts and some spices. c) Others: These types of fungi are belonging to several genera and have been isolated and characterized from spoiled milk and food; they are able to produce mycotoxins such as *Fusarium* species **(Pal, 2014).**

4. Conclusion

The contamination of milk and milk products by pathogenic bacteria and spoilage producing microbes result in great financial loss to the dairy sector. Bacterial contamination occurs either by direct transfer from the blood due to systemic infection (endogenous contamination) or by contamination by faeces, skin, utensils and environment during and after milking (exogenous contamination). The main undesirable contaminants of milk in the refrigerated dairy food chain are psychrotrophic bacteria. From the previously mentioned contamination sources measures should be taken to avoid and prevent the spread of zoonotic diseases among animals, improving their hygiene, controlling the infection from feed and fodder, safe waste management and easy access to veterinary service. Investigators reported that most pathogenic as well as spoilage microorganisms can be unable to grow in milk and milk-products when the pH of the environment is 4.5 or lower. These microbiologically safe products would contribute towards the nutrition of susceptible infants to diarrheal diseases. The dairy-borne diseases in public health programs are of importance in the surveillance of milk food borne diseases by monitoring microbial contamination and milk borne pathogens in milk products. Proper governmental policy for the assurance the quality of milk and dairy products should be implemented to reduce the public health risks towards these products. More studies should be conducted concerning the inactivation kinetic determination to establish how the process conditions for microbiological safety should be done.

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Declaration of interest

Author declares that he does not have any conflict of interest.

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