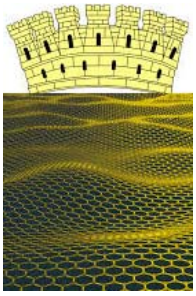

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Review

2014 SPREAD/UNCONTROLLED EBOLA OUTBREAK

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ABSTRACT

It is interesting to determine the underlying factors contributing to the emergence, rapid spread and uncontrolled nature of 2014 virus outbreak, the first to display a distinct epicentre in West Africa. Novel treatment and precautionary approaches are explored. The model of Ebola virus transmission dynamics is reviewed, with the aim to provide a broad sketch of the fundamental human–Ebola-virus biophysical forces that enable and constrain EVD. Kumar group reported a model of Ebola evading the immune system. What are the factors of the emergence, rapid spread and uncontrolled nature of 2014 virus outbreak? How to treat EVD?

Keywords: Ebola virus disease, seasonal distribution, deforestation, socio-political infrastructure, micronutrient deficiency.

1. INTRODUCTION

Two thirds of human pathogens are of zoonotic origin, *e.g.*, Ebola (identified in 1976), human immunodeficiency virus (HIV). A pathogen spread relies on perpetual contact with new groups of susceptible individuals. Ideal conditions for zoonotic-viruses emergence and spread are socio-economic/environmental changes, long-distance mobility (air travel) and changing climate. Ideas in biology should be valued by number of questions they generate. Questions (Qs), answer (A) and hypothesis (H) on Ebola virus disease (EVD) follow.

Q1. How is EVD contagious?

A1. It passes on by contact with secretions of a patient that be in active phase, *i.e.*, fever.

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Q2. What does it happen at the host/pathogen interface?

Q3. Why are certain viruses capable of jumping to new species?

H1. The genetic plasticity [ribonucleic acid (RNA) polymerase has no proofreading activity and is highly error-prone] is key if the virus is to overcome a host immune attack.

Q4. Is EVD a growing threat?

Q5. What will happen if phase-3 trials have insufficient power to determine efficacy?

Double-stranded (ds)RNA, an intermediate in viral replication, triggers release of cytokines, primarily interferons, which in turn causes upregulation of antiviral genes and antibodies. Viruses developed ways to inhibit interferon response: interferon antagonists [in Ebola, viral membrane-associated (VP24) and polymerase complex (VP35) proteins].

Mas-Coma and González-Candelas raised questions on EVD arrival at Spain on 2014 [1].

Q6. What are causes of the infection?

Q7. What is the risk for the health staff?

Q8. What is the possibility of an outbreak in Spain?

Q9. Is there a possibility that the virus mutate and become its transmission by the air?

Q10. Is it necessary to put to sleep the dogs of infected people?

Martín-Moreno raised the following questions on 2014 EVD outbreak [2].

Q11. What steps must one take before potentially infected people?

Q12. What is the alert level that can have a country as Spain?

Q13. Does the Law of Labour Risks observe the protocol?

Q14. Why has this infection happened?

Q15. What has it happened?

Q16. Was there a human error?

Q17. What health action must be activated after an infection?

Q18. The suits that were used, are the adequate ones for this type of virus?

Q19. Had a suit of higher-level protection better covered the exposure to the virus?

Q20. What information must be provided to worried neighbours?

In earlier publications it was reported anthropoid-apes phylogeny [3], fractal [4]/hybrid-orbital [5] analyses of protein tertiary structure, tumour‑immune cells competition [6], information-entropy classification of molecules [7–9], classification, diversity, complexity and emergence [10–12], periodic classification of HIV inhibitors [13], molecular classifications of thiocarbamates with cytoprotection activity *vs*. HIV [14], styrylquinolines as HIV integrase inhibitors [15] and *N*-aryloxazolidinone-5-carboxamides as HIV protease inhibitors [16], hypothesis on how acquired immunodeficiency syndrome (AIDS) destroys immune defences [17], interrogation of molecular structure [18], hyaluronan as a drug delivery vehicle [19,20]. In the present report, the model of Ebola virus transmission dynamics is reviewed, with the aim to provide a broad sketch of fundamental human–Ebola-virus biophysical forces that enable and constrain EVD.

2. 2014 EBOLA VIRUS DISEASE OUTBREAK

Elena raised the following questions on 2014 EVD outbreak [21].

Q1. Are there equipment and experience needed to treat EVD patients?

Q2. What is a virus?

Q3. What factors do favour the appearance of new viral diseases?

Q4. Why is it difficult to control infections or find vaccines *vs*. viruses-caused diseases?

Q5. How does one interfere a virus without doing anything to the cell?

Q6. Why have people no vaccine?

Q7. Ebola virus was identified in 1976 but, why is the most virulent outbreak nowadays?

Q8. Could the epidemic be spread to Europe?

Q9. Could an epidemic begin?

Q10. Double level. Must one be critical with protocols followed by Spanish Government?

Q11. To put to sleep the dogs of infected people?

Q12. Does it represent a danger or an opportunity?

Q13. Is there the equipment needed to have a dog infected by Ebola?

Q14. Are the media sensationalist?

Q15. Laying on of hands, seawater, essential oils of cinnamon/oregano, Ag^{+} or ozone therapy *via* rectal are some of the supposed cures *vs*. EVD. How must one act *vs*. the calls?

Q16. In who have they tested them?

Q17. With what probability do they function?

Q18. Are people close to eradication of viral diseases or is it a long way to cover?

3. ZOONOSES

3.1. Ecology of Zoonoses: Natural and Unnatural Histories

Karesh *et al*. proposed Qs and Hs on ecology of zoonoses (*e.g*., EVD, original HIV) [22].

Q1. How do zoonotic diseases result from natural pathogen ecology?

Q2. How do other circumstances (*e.g*., animal production, natural-resources extraction, antimicrobial application) change the dynamics of disease exposure to human beings?

Q3. Where does one stand in zoonoses and marginalized infectious diseases of poverty?

Q4. How do these pathogens survive and change?

Q5. Why do pathogens what they do?

H1. H of competitive exclusion. The ecological principle of competitive exclusion is the basis for common approaches to control of zoonotic pathogens in livestock and poultry.

Q6. What techniques are animals slaughtered and processed with?

Q7. How are products stored, packed, transported and prepared at the consumption place?

H2. Antimicrobial-resistance genes originated as evolutionary response to drugs produced by free-living bacteria, fungi and plants to protect from infection or competition.

H3. (Bennett). Resistance patterns and genes are much the same in wildlife and livestock.

H4. Whatever sources of resistant bacteria/genes, differences in wildlife-species ecology (*e.g*., diet, physiology) cause selection pressure on microbes, rather than differential exposure to anthropogenic antimicrobials or presence of different resistant strains in environment.

Q8. Has the use of antibiotics in agriculture exacerbated drug resistance in people?

Q9. What is transfer extent of antimicrobial-resistant organisms from animals to people?

Q10. What is the potential for reversal of resistance?

Q11. Would it occur in clinical settings after a change in antimicrobial use?

H5. Reversion to drug susceptibility depends on occurrence of natural dilution of microbial populations with susceptible strains and fitness costs of resistance.

H6. No approach to search for potential new human pathogens, *e.g*., tracing back source host of a human disease, would identify simian immunodeficiency virus (SIV) as a risk.

Q12. How is the environment changing?

Q13. How do these changes affect microbial dynamics across the system?

H7. Enhancing the role ecologists play in control programmes includes model-outputs production by collaboration with clinicians with real-time data, participation in prospective and retrospective study design, and field studies to identify risk factors to target surveillance.

H8. Disease ecology drives advances in prediction of novel-zoonoses emergence/spread.

Q14. How can dynamics of a pathogen in a wildlife host change seasonally?
Q15. How does it function the microbiome from people?
Q16. How does it function the microbiome from animals they contact?
Q17. What does it cause zoonotic microbes to proliferate in some conditions?
H9. One Health approach provides a wider, holistic view with which to achieve this aim.
Q18. Where do zoonoses occur?
Q19. How do zoonoses occur?

3.2. Drivers/Dynamics/Control of Emerging Vector-Borne Zoonotic Diseases

Kilpatrick and Randolph proposed Hs/Q on control of zoonotic diseases [23].

H1. Climate change leads to more widespread and abundant vector-borne pathogens (VBPs) as more of the planet starts to resemble closely the tropics.

H2. The arrival of exotic and upsurges of endemic VBPs are because of climate changes.

H3. Effects of climate change on VBPs are variable, as expected from complex systems.

Q1. What will global warming do?

H4. Feeding on additional alternative hosts results in risen vector densities, which result in higher transmission even if a smaller proportion feed on people.

H5. Dilution effect. Natural biodiversity diverts vectors from infectious hosts.

3.3. Prediction and Prevention of the Next Pandemic Zoonosis

Morse *et al*. proposed Hs/Qs on prediction/prevention of next pandemic zoonosis [24].

H1. Efforts to co-ordinate global strategy to fight pandemics are timely and important.

H2. Disease emergence is driven by anthropogenic changes, *e.g*., agriculture expansion.

H3. Human populations are exposed to a wide variety of non-human-animal pathogens.

H4. Viruses have the potential to evolve more rapidly than do other kinds of pathogen.

H5. Simple behavioural precautions greatly reduce risk.

H6. Risks to hunters, food handlers and livestock workers from occupational exposure are reduced in hotspots of emerging infectious diseases though routine sanitation and biosafety precautions, as was tried with A/H5N1 flu in agricultural settings.

H7. Nosocomial spread is prevented by infection control practices, *e.g*., sterile injections.

H8. Viral-relatedness analysis as a predictor of emergence. Wildlife viruses that are more closely related to known human pathogens are more likely to infect people than not similar.

Q1. How do viral traits/phylogenetic relations correlate with pathogenicity?

Q2. Why have some pathogens a high propensity for host jumps?

Q3. Why do some viruses that are benign in their natural hosts induce a severe or lethal hyperinflammatory response in a new host (*e.g*., Ebola, sin nombre virus)?

H9. Strong patterns of co-evolution during recent evolutionary time indicate stable long-term interactions with little host-switching, but pathogens that frequently moved from one host to another have poorly aligned co-evolutionary trees.

Q4. How feasible is a programme for identification of the many thousands of novel pathogens that are probably in wildlife globally?

Q5. How to address the underlying drivers that are essentially ecological (*e.g*., livestock production–wildlife populations juxtaposition) or occur on large spatial scales because of economic activity (*e.g*., change in land use related to development of tropical forests)?

Q6. Could the seemingly opposing forces of economic development and public health be reconciled before rather than after these outbreaks occurred?

H10. Expansion of so-called health impact assessments is an approach.

H11. Incentives for industries propagating pandemics is linked to development initiatives.

H12. Efforts to curtail wildlife trade for food and pets in hotspot and other countries include consumers incentives creation that lead to certification of healthy-practices industries.

Q7. Can researchers intervene before a pathogen reaches human population?

Q8. How can researchers intervene before a pathogen reaches human population?

4. VIRAL VIRTUOSOS OF PERSISTENT *VS*. ACUTE INFECTION

Sullivan raised some questions on viral virtuosos of persistent *vs*. acute infection [25].

Q1. How do viruses orchestrate lifelong infections?

Q2. How do human-body viruses use micro (mi)RNAs and their own to infect?

Q3. How could miRNAs/noncoding (nc)RNAs function spawn therapies, while yielding insights into evolutionary forces dictating parasitism/mutualism/multiorganismal relations?

Q4. Does RNAinterference (i) serve as a meaningful antiviral response in mammals?

Q5. How does the human virome contribute to health and disease?

5. VIRUSES AND CELL DEATH PROGRAMMES

Lamkanfi group raised a question on the regulation of apoptosis during infection [26].

Q1. How do viruses evade host cell apoptosis?

Kaminskyy and Zhivotovsky revised/proposed a Q/H on cell-death consequences [27].

Q2. How do viruses interact with the cell death machinery?

H1. Necrotic cell death is regulated by a specific set of signal transduction pathways.

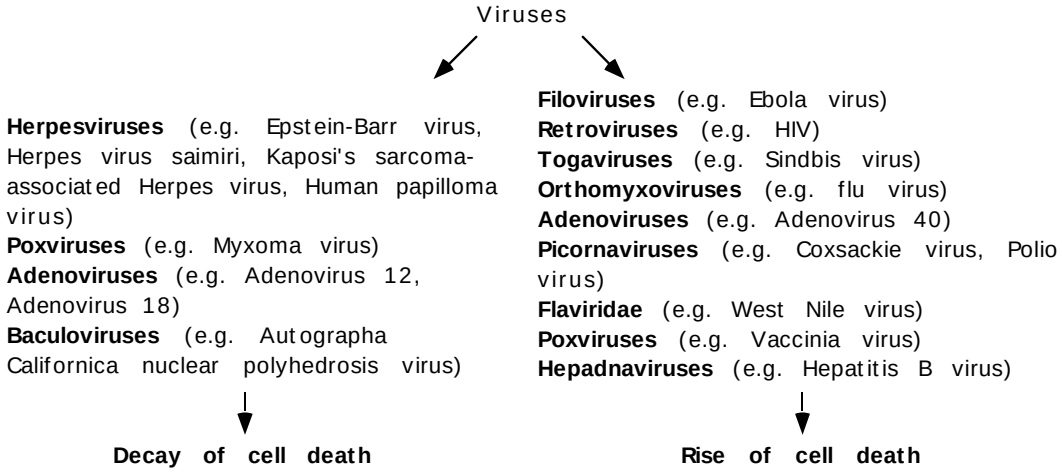
Figure 1 shows cell death-related consequences of viral infection.

Q3. How does the Ebola virus induce massive apoptosis of lymphocytes?

H2. Inflammatory mediators/NO secreted by macrophages induce bystander cell death.

H3. Viral proteins induce lymphocytic cell death.

Figure 1: Cell death-related consequences of viral infection



6. INTRACELLULAR EVENTS/CELL FATE IN FILOVIRUS INFECTION

Mühlberger group proposed Qs/Hs on intracellular events/cell fate in this infection [28].

Q1. What is known about intracellular events leading to virus amplification in infection?

Q2. How may cellular dysfunction and cell death correlate with disease pathogenesis?

H1. Adaptive immune responses may occur.

H2. Lymphopenia from apoptosis contributes to failure to clear infection.

H3. A well-regulated cytokine response early in infection is critical to disease outcome.

Q3. How do filoviruses enter, replicate and assemble exploiting cellular machineries?

Q4. How do filoviruses interact with cellular signalling pathways?

Q5. What is the current understanding of non/infected-cells fate in filovirus detection?

Q6. What changes are in ultrastuctural data of non/infected cells, in infection?

Q7. Why does little or no inflammatory cellular response occur at viral-replication sites?

Q8. What does it happen in the infected cell?

H4. The folate receptor is a significant filovirus receptor.

H5. Thymus-dependent lymphocyte (T-cell) immunoglobulin and mucin domain-1 (TIM-1) is a receptor for Ebola and Marburg viruses type-I transmembrane glycoprotein (GP).

Q9. What is the fate of infected and non-infected cells in filovirus infection?

Q10. How do viruses interact with the cell death machinery?

H6. In extensive filovirus infection, cells are unable to keep a normal water‑ion balance.

Q11. Do filoviruses manipulate signalling pathways in apoptosis or cell survival?

H7. Inhibition of retinoic acid-inducible gene-1 (RIG-I) by VP35 stops apoptosis.

H8. Cells using Toll-like receptors (TLR)-mediated antiviral pathways, *e.g.*, plasmacytoid dendritic cells (DCs), are less prone to the inhibitory effects of VP35 Ebola virus than cells relying on RIG-like signalling pathways.

H9. The GP-induced cytotoxicity is caused by GP‑guanosine-5'-triphosphate (GTP)ase dynamin interaction, interfering with intracellular trafficking of cell surface proteins.

Q12. What is the involvement of dynamin in GP-induced cytopathic effect (CPE)?

H10. Extracellular signal-regulated kinase-2 (ERK2) is involved in CPEs induction.

H11. A mechanism of cytotoxicity is induction of endoplasmic-reticulum (ER) stress.

Q13. Are the different proposed mechanisms to explain GP-mediated CPE connected?

Q14. How are different proposed mechanisms to explain GP-mediated CPE connected?

Q15. What is the ability of GP to induce cell death?

Q16. What does it happen in the infected cells?

H12. Host-derived proteins contribute to cluster of differentiation (CD_4) T-cell death.

H13. An accumulation of incomplete viral transcripts during abortive infection of resting CD_4 T-cells activates intrinsic pathways, which lead to apoptosis during HIV infection.

H14. Generalized mechanisms contribute to lymphocyte apoptosis in filovirus disease.

H15. Both intrinsic and extrinsic apoptotic pathways contribute to lymphocyte depletion.

H16. Loss of lymphocytes contributes to failure to generate adaptive immune responses.

H17. Dysregulated DCs/macrophages contribute in other ways to lymphocyte apoptosis.

H18. Programmed death-1 (PD-1) signalling results in decayed T-cell proliferation because of induction of apoptosis *via* PD ligand-1 (PD-L1) binding.

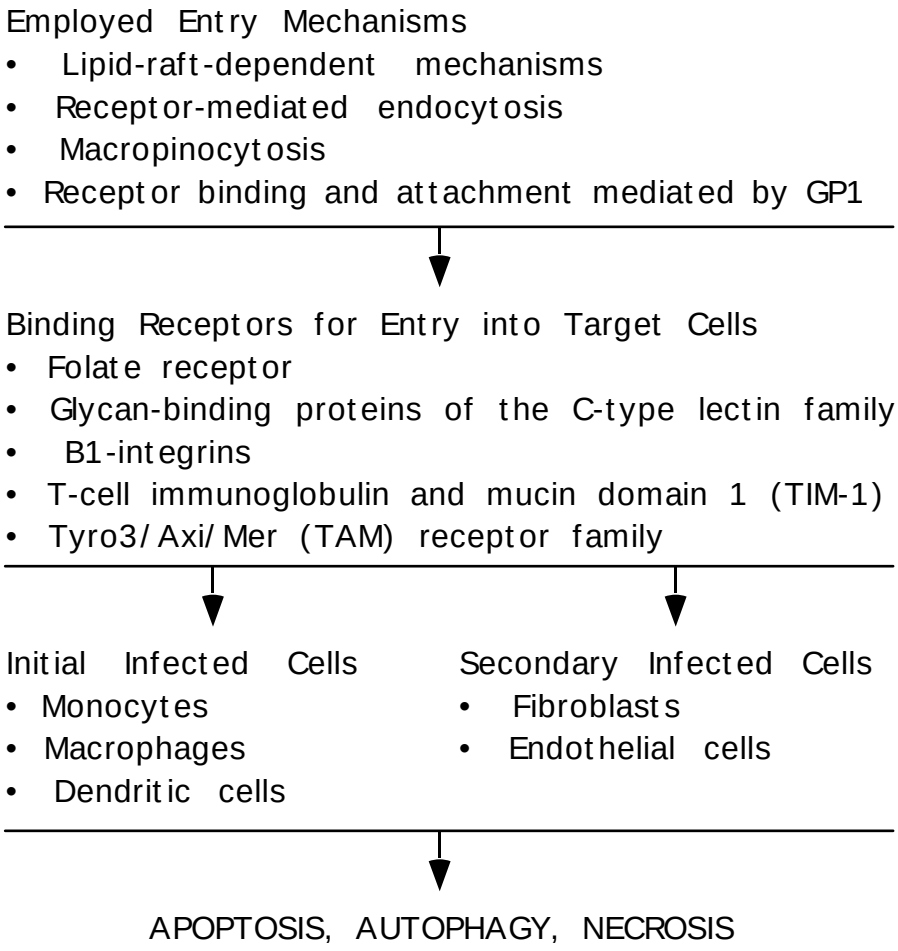
7. EVADING THE HOST'S IMMUNE RESPONSE

Kumar group reviewed emergence of 2014 EVD outbreak and proposed Qs and Hs [29].

Q1. What factors are contributing to emergence, rapid spread and uncontrolled nature?

Q2. How to treat EVD?
Q3. How does the Ebola virus replicate?
H1. Different genes related to early immune response to virus, impart bats resistance.
H2. Bat genes co-evolve with virus genes.
Q4. Could redirection of non/human primates immune system reduce Ebola's death rate?
Q5. Why have bats evolve to resist fatality in the face of the Ebola virus?
Q6. How have bats evolve to resist fatality in the face of the Ebola virus?
H3. The virus strain in 2014 epidemic shows signs of genetic mutations.
Q7. Did Ebola Zaire exist in Africa before 1976, or evade detection and documentation?
H4. All emergences between 1976 and 2005 are descendants of Yambuku-like virus.
H5. The descendents spread *via* outbreak regions.
H6. Ebola Zaire employs a mechanism of spread.
H7. Ebola Zaire-West African virus has a substitution rate 8×10^{-4} per site, per year.
H8. Outbreaks are indeed representative of independent zoonotic transmissions.
H9. The VP24 is critical in contributing to virulence and plays a role in host adaptation.
Q8. How does Ebola evade the immune system?
Mononuclear phagocyte system is the first one to be manipulated by virus (*cf*. Fig. 2).

Figure 2: Ebola evades the immune system



H10. Higher virulence levels exist in initial cases rather than infection successive waves.
Q9. What is the cause for risen initial virulence and subsequent risen chance of survival?
H11. Currently 22 million Central and West Africans are at risk of an Ebola infection.
H12. Previous H. Strain virulence is solely a product of viral genetics.
H13. Socio-political/environmental climates create flux in mortality rates and spread.
H14. The second outbreak was initiated by an ill imam traveling from Guinea to Mali in an attempt to receive better care at the Pasteur Clinic in Bamako.
H15. The 2013-2014 epidemic varies in trends and final outbreak size between Sierra Leone, Guinea and Liberia, which share common cultural and geographical traits.
H16. Bats are natural reservoir of Ebola and have behavioural associations with seasons.
H17. A trigger in the 2014 outbreak is the shift in the seasonal migratory route of bats.
H18. Ebola Zaire virus creates Se demand on infected host, depleting hosts stores in days.
H19. Survival of Ebola virus *via* replication is increased by low Se concentrations.
H20. Disabling T-cell from Ebola Zaire is similar to Se-deficient hosts infected with flu.
H21. Fibrinogen levels, procalcitonin and cross matching of blood assays are unsafe.
H22. Colloid solutions, human albumins and synthetic starches are associated with adverse renal outcomes and provide no benefit to EVD infected patient.
Q10. A dead-end host: Is there a way out?
Q11. Why was not Zmapp made more widely available in Africa?
H23. Ethical H. Administering an experimental drug without safety data is unethical.
H24. An advantage to the blood-transfusion recipient exists if the donor is from the same geographical region as EVD infected.
H25. Health care workers treating EVD outside treatment units are at an increased risk.
Q12. What role should pharmacy play in epidemics like this?
Q13. Ethical Q (EQ)1. Why to transport aid workers to native countries for treatment?
Q14. EQ2. What are the benefit *vs*. risk profiles of experimental therapies?
Q15. EQ3. Is practice of rushing an experimental agent to control an epidemic adequate?
Q16. EQ4. Is drug impact on transmission/containment of an epidemic positive/negative?

8. DISTINCT LINEAGES OF EBOLA VIRUS IN GUINEA: 2014 EPIDEMIC

Simon-Loriere *et al*. raised questions on distinct lineages of Ebola virus in Guinea [30].
Q1. What rate did Ebola virus evolve during the West African outbreak of EVD?
Q2. What may this mean for the virus adaptive capacity, *e.g*., changes in virulence?

9. DISCUSSION

Ebola is a dangerous virus that could cause in people a grave disease, which could reach the dead. The virus comes from Africa, where it causes the greatest problems. The population of the entire world is worried by EVD, and took measures to contain it and trait the persons that be infected. No signal exists that EVD expand widely to other continents. The first time that EVD was described was in 1976 and the most surprising of the virus is that it is aggressive: it has a mortality ranged in 40–90% (in Ebola Zaire strain, 85–95%). In the future, a holistic examination of the full spectrum of viral diversity, not just of the viruses that make humans ill, may be factored into medical decisions; *e.g*., a rise in the levels of a benign persistent virus was suggested as an indicator for monitoring therapeutic immune suppression. Beneficial altering host viral communities, so-called provirotics, could

contribute to improved human health. Advancing the notion will require effort to catalog and exploit the human virome, and decipher the contributions of the humble but elegant persistent viruses (and ncRNAs that regulate them).

Humans are not prepared for viral outbreaks: (1) the state of knowledge about host–pathogen interactions is selective; (2) a deficit of trained medical and scientific personnel delays deployment to the established Ebola treatment centres in West Africa; (3) the public is unaware of the threat of emerging viral diseases. Funding for basic research in virology is insufficient. The Ebola outbreak remembers people that a more thorough understanding of zoonotic viral infections is necessary, especially in the face of the changing environment. Interferon research is an emerging avenue, which helps achieving the understanding and improving the quality of viral infection management.

Genomic surveillance is a complement to local epidemiological research. Deployment of additional next-generation sequencing (NGS) facilities in West African surveillance net, avoiding the logistical and regulatory hurdles associated with long-distance sample transportation, will contribute to control the current epidemic and help limit future outbreaks.

10. CONCLUDING REMARKS

From the present review and discussion the following remarks can be drawn.

1. Safe-practices guidelines, *e.g*., ecology to reduce disease risk, are needed.

2. New understanding of noncoding ribonucleic acids may solve a long-standing puzzle about how viruses orchestrate lifelong infections. Microribonucleic acids can be thought of as tools that viruses use to manipulate key aspects of human biology and immune response, in order to hitchhike with people throughout their evolutionary history. The co-existence of virus and host involves careful control of the viral life cycle: whereas Ebola virus infection is flashy, persistent infection is elegant. Viral microribonucleic acids can optimize the location and timing of virus replication to fly under the radar of the host immune response.

3. Lesson to be learned: How little one knows about why and how certain viruses spill over from their natural hosts, and how they interact with the human immune system.

4. Transmission among humans occurs *via* blood and body-secretions exchange. Triggers in outbreaks are low temperatures, high humidity, seasonal change and its relation with animal behaviours, socio-economic decline leading to deforestation, *etc*. Contributing to success is delayed identification of outbreak, which initially spread unsuspectedly to neighbouring regions. While multiple candidate vaccines and antiviral therapies are in development, preventative public health interventions (*e.g*., risk communication, protective-principles implementation) are a means to mitigate spread of Ebola virus disease.

5. While one is alive, he is subjected to threats, which could kill him. However, to live frightened by the dangers, when the reply does not depend on oneself, is little recommendable. It is essential to get the needed means to control the outbreak in its origin.

6. Trials would need to reassume rapidly when and where the next Ebola outbreak occur.

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Article

ECOLOGICAL RESTORATION STUDIES OF SOME BIOLOGICAL INERT, HAZARDOUS WASTE

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ABSTRACT

In this study, the results of research on ecological restoration of a deposit of 150ha of fly ash and boiler slag are presented. The nutrients for plant growth were done with organic fertilizer, slaughterhouse sludge. Stimulation of vegetation was done with an agent based on brown algae extract. The study was conducted over two successive years. Plant growth was performed in fertilization variants with optimum amount of10t/ha slaughterhouse sludge with growth stimulus. The stimulus advantages have been the increase of seed amount of up to 37% and reduction of plant toxic metal content *i.e*. for Pb with 62.1%.

Keywords: fly ash, boiler slag sludge, slaughterhouse, ecological restoration.

1. INTRODUCTION

Waste disposal on uncovered deposits is a usual way to remove it from anthropogenic activities areas. Waste deposits are located in the vicinity of sources: *i.e*. fly ash and boiler slag deposits from power. The studied lignite fly ash and boiler slag deposit is a plain deposit that occupies an area of 150 ha. It has a height of 500m and a capacity of over 4.8 million cubic meters. It is located at a distance of 1.5-4 km from inhabited areas. To protect the environment numerous arrangements were conducted: a well compacted clay base layer of 3.5-6.5 m to limit water percolation, perimeter dams of the storage area for limiting the scattering of particles by erosion, systems for drainage, collection and storage of water. The deposit contains three compartments that fill up successively. The deposit has operating permit under the laws of Romania. With all the arrangements made, the deposit is an area

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with potential danger to dissipate through deflation lightweight materials in the neighbouring agricultural areas, to gravely alter the ecosystem, and strongly alter the landscape etc.

Fly ash and boiler slag deposits in Romania contain mostly aluminium, silicon, calcium, iron, magnesium, sodium, phosphorus and sulphur, and do not retain water in the upper layers [1, 2]. They are biologically inert. Carbon content is due to partially burned coal and is 0-15% range [3]. Nitrogen content is extremely small, and dependent on the type of coal burned [4]; its concentration in charcoal is between 0.3-3% and decreases by combustion to 0.01-0.1%. The maximum range of the nitrogen content of slag and ash may be due to partially burned coal, or can be of atmospheric origin [5, 6]. Ashes are inert, lacking any life form. The proliferation of microorganisms is hampered by unfavourable conditions of humidity, temperature, nutrition, in the upper layers. From the older layers of fly ash and boiler slag microorganisms were isolated but they are present only in the spore form [6]. When the deposit is not administered, then the field can develop weed species that tolerate the hostile environment but by their spreading can affect negatively the neighbouring crops. Moreover, selected plant species can be planted directly. But this is a risk, because the chemical and physical characteristics of the soil can seriously affect plant growth.

Therefore, organic amendments are added to support plant growth. Partially dehydrated municipal sludge, different composts, chicken manure, green compost and others can be used. These materials are mixed with 5-10 cm surface layer of ash to form a layer of fertile, artificial soil. Many researchers show that these used organic substances promote an ecosystem in the formed layer and support microbial communities necessary for the formation of plant growth conditions. The positive effect of organic amendments is endorsed by a number of researchers [6, 7]. The role of these materials in the recovery of damaged areas is demonstrated in many countries [8-10] both for grassland plants and specific shrubs. Vegetation strategy relates generally to the improvement of the physical, chemical and biological characteristics of the destroyed land for vegetation: low nutrient content, inappropriate texture, minimized capacity of water retention, high content of heavy metals etc. Selection of plant species is an important factor because they must tolerate pedoclimatic regime of the deserted area. From the experience of many researchers, the most tolerant plant species are those from the Gramineae and Leguminosae families. [11, 12]

In this study, the results obtained regarding: 1. The recycling of nutrients embedded in biodegradable organic sludge, slaughterhouse sludge; 2. Environmental protection against the dispersion of pollutants, the restoration of damaged ecosystems and landscape near the city; 3. Obtaining seeds and biomass with reusable possibilities, are presented.

2. METHODS

The experimental study was done in the experimental bloc with three variants: va s1, va s 2, va s 3. The experiments were carried in pots. The pots were fitted with inert fly ash and boiler slag of thermal plants and fertilized with slaughterhouse sludge in quantity of 5.0, 10.0, respectively 25.0 $t \cdot ha^{-1}$ and bacterial stimulus *Bio complex 900*, provided Ekogea Slovenia. Control variant – pot, va s2, was fitted with boiler slag and fertilized with 5 $t \cdot ha^{-1}$ sludge without algae addition. Each variant is done in three replicates. The quantity of metals from the boiler slag used in this study is presented in Table 1.

Table 1. The content of heavy metals from the fly ash and boiler slag used.

pH	*Heavy metals from the fly ash and boiler slag used [mg·kg^{-1} D.M.]							
	Cd	Cr	Cu	Fe	Mn	Ni	Pb	Zn
7.11± 0.2	2.7 ±0.9	75.0 ±4.6	62.1 ±.2	2060.0 ±53.3	238.4 ±5.8	85.1 ±2.9	35.2 ±0.8	169.2 ±6.2

***** Values are means of 12 samples

The slaughterhouse sludge characteristics used in this study is presented in Table 2.

Table 2. The slaughterhouse sludge characteristics

The slaughterhouse sludge characteristics				
pH	Humidity[%]	Organics [%]	Total nitrogen [%]	Total phosphorus[mg·kg^{-1} D.M.]
7.1	71.86	90.77	2.314	1126±55

Determination of soil metals (to unfertilized/fertilized fly ash and boiler slag), i.e. Cd, Cu, Cr, Ni, Pb, Zn was done according to SR ISO 11047-99. The principle of the method consist in aqua regia metals extraction according to ISO 11466-99. Iron determination in soil samples was made in accordance with ISO 11466- 99 SR. Soil extract analysis was done by atomic absorption photometry in accordance with SR 13315-96/C91:2008. It was used an Atomic Absorption Spectrophotometer, GBC Avanta AAS, GBC Scientific Equipment Ltd. Company. Total nitrogen determination was performed according to ASTM D 5373-08, SR ISO 10694-98 and STAS 398-92. Phosphorus determination was performed according STAS 12205-84.

During sowing, the topsoil - fly ash and boiler slag - was watered with a mixture of brown algae extract and water (ratio 1:50). The experiment was carried out in pots with 6.5 kg of soil. In the pots, 20 g/pot of seeds from the *Lolium perenne* plant species were planted. The species tolerates pedoclimatic conditions specific to the western part of Romania. Plants were seeded in the spring. Pots were placed in the open and watered periodically. In the first year of culture, plant samples were harvested between July and October, every two weeks. In the second year of culture, plant samples were harvested in May-July, every 2 weeks. During August – September, cultures were not harvested to allow their fructification. Seed biomass harvesting was performed at the end of September. Metal analysis was done for the aerial parts and seeds of the plants obtained in the 2nd year of culture. Heavy metals of tissues of mature plant was determined and compared with Romanian standard. Plant tissues were thoroughly washed 3x 25ml with de-ionized water, at room temperature, to remove any soil particles attached to plant surfaces. Plant sampling was performed in agreement with the standardized methodology (the methodology described in STAS 9597/1-74, and the sample analysis was done in accordance with STAS 9597/17-86): 5g plant tissues were dried to constant weight at 105 °C. Plant samples with precise weight are then brought to 550°C; to the residual materials 3.5ml of concentrated hydrochloric acid (d =1.189 kg·L^{-1}) are added, samples are maintained 30 minutes on the dry sand bath. Then, 1.5ml hydrochloric acid (d =1.189kg·L^{-1}) : de-ionized water, 1:1 solution was added. After very slowly filtering on a paper filter (filtration smooth type 640de Mackerel-Nagel Germany), the samples were taken to calibrated flasks (25mL) with hydrochloric acid (d=1.189kg·L^{-1}) : de-ionized water, 1:1 solution. Heavy metals content from plant tissues extracts analysis was determined using an

Atomic Absorption Spectrophotometer, GBC Avanta AAS, GBC Scientific Equipment Ltd. Company. The detection limit of the device for Cd is 0.0431mg/L, for Cr - 0.0386mg/L, for Cu - 0.0349mg/L, for Fe - 0.0408mg/L, for Mn - 0.0421mg/L, for Zn is - 0.0358mg/L, for Pb - 0.0393mg/L for Ni - 0.0446 mg/L.

3. RESULTS AND DISCUSSIONS

The plants sprouted at 2-3 weeks after sowing. Vegetation coverage at 30 days after seeding was 80-90%. After this period, the plants sprung on the experimental variant fertilized with slaughterhouse sludge in the absence of the stimulus dried in a proportion of 55%. The remaining plants showed signs of suffering (yellowing and drying of basal leaves). In the second year of culture, the green cover grown on the experimental variant fertilized with slaughterhouse sludge in the absence of the stimulus was restored.

3.1. Results

Table 3 shows the amounts of biomass harvested periodically from the aerial parts of the *Lolium sp.* plants. In the first year of culture, the amount of harvested green mass was similar in all variants fertilized with slaughterhouse sludge in the presence/absence of growth stimulus. It is noted that in the second year the amount of harvested biomass was 40.9-57.2% higher than in first year of culture. This increase demonstrates the gradual adaptation of plants to the conditions imposed by this experiment. The amount of biomass harvested in the first year of culture was influenced by the amount of fertilizer used. The optimal amount of fertilizer for obtaining a high yield of biomass was 10t/ha. The addition of growth stimulus in the first phenological phases of development resulted in an increase to 10% vs. crop biomass resulting from the variant fertilized in the absence of the stimulus.

Table 3. The amount of biomass harvested from the aerial parts of plants from *Lolium sp*.

No	Variant	Harvest [g /pot]		Harvest increase, second year vs. first year. [%]	Total harvest [g /pot]
		first year of culture	the 2nd year of culture		
1	va s1	170.0±2.5	392.0±32.7	56.6	462.0±39
2	va s2	181.0±2.9	423.0±35.9	57.2	604.0±40
3	va s3	180.2±2.8	305.0±29.7	40.9	485.2±35
4	va 2	177.0±2.8	369.0±31.5	52.0	546.0±29

Table 4 shows the seeds quantities harvested from the experimental variants. It is seen from Table 2 that the use of optimal dose of fertilizing agent in combination with growth factors previously determined, of 10t/ha, increased the amount of seeds by 37% vs. the quantity of seeds harvested from the same fertilized variant in the absence of the stimulus. This stimulating agent positively influenced the quantity of seeds obtained in the 2nd year of culture.

Table 4. The quantities of seeds from fly ash and boiler slag cultivated with *Lolium perenne*

No.	Harvest	Amount of seeds from [t·ha^{-1}]			
		without stimulus	with stimulus		
		va 2	vas 1	vas 2	vas 3
1	Seed Second year	1.35± 0.05	1.4± 0.04	2.13± 0.07	3.60± 0.09

Table 5 shows metal quantities that have accumulated in the green biomass harvested in the second year of culture, in the aerial parts.

Table 5. Metal quantities accumulated in the green biomass harvested in the second year of culture, in the aerial part

Variants	Metal content [mg·kg^{-1} D.M.]							
	Cd min - max	Cr min - max	Cu min - max	Fe min- max	Mn min- max	Ni min - max	Pb min - max	Zn min - max
va s1	0.35-0.54	1.68-1.77	1,40-1.57	30.6-36.3	22.2-33.7	1.35-1.55	6.34-7.14	11.7-15.7
va s2	0.37-0.64	1.78-1.90	1.67-1.70	39.5-44.5-	34.0-38.8	1.45-1.67	5.09-5.20	18.0-23.7
va s3	0.39-0.57	1.90-2.07	1.85-1.95	46,0-48.2	30.0-36.8	1.37-1.71	5.00-6.77	17.4-19.0
va 2	0.45-0.67	1.92-2.13	2.70-2.96	70,0-82.9	59.9-67.2	1.54-1.83	11.9-13,2	22.3-25.8
Maximum Romanian limit for organic matter with 12% humidity [13]	1.0	*	*	*	*	*	100	*

* Without analyzing

3.2. Discussion

It is seen from the tables that the aerial part of the plants harvested from fertilized variants in the presence of the stimulus, have accumulated small amounts of toxic metals, such as: Cd, Cu, Cr, Ni, Zn, Mn and Fe. Instead, plants accumulate large amounts of lead, 5.0-7.14 g • kg^{-1} D.M. It is observed from the table that plants grown on variants fertilized and treated with stimulus have accumulated lower amounts of metals than plants grown on control, fertilized in the absence of the stimulus. Thus, the accumulation of Pb decreases by 45.9-62.1% vs. the bioaccumulation in plants grown on variants fertilized in the absence of the stimulus. With all this Pb content reduction in plant crops in the second year of culture,

plants will be used as compost add so that by mixing they will decrease the content of this metal to the limits allowed by compost law. Seeds harvested from the herbaceous plants culture resulted in the second year can however be used for next sowings. It is necessary that all crops resulting from the slag and ash fertilized with slaughterhouse sludge in the presence/absence of a plant growth stimulus to be closely monitored. Plant crops can be used in animal feeding only if the bioaccumulation of metals does not endanger their health.

4. CONCLUSIONS

The strategy of ecological vegetation/restoration of fly ash and boiler slag, biologically inert, non-hazardous deposits with slaughterhouse sludge regards the improvement of the physical, chemical and biological properties of fly ash and boiler slag deposits in order to facilitate plant growth. Selected plants of the *Lolium perenne* species are an essential factor in restoring the landscape as they quickly form a stable and sustainable vegetation cover. Use as a fertilizing agent of the slaughterhouse sludge provided the basic compounds of C, N, P, for a lasting development.

The addition of organic Many thanks are due to Prof. Nicolae Dragomir at Banat University Agricultural Sciences and Veterinary of Medicine, from Timisoara, Romania and SC. EKO GEA EAST SR. Romania. The research was given by the Ministry of Education Research, Youth and Sports which provide financial support for Nuclear program ON 09-13.based on marine brown algae in combination with fertilizing agent resulted in resistant crops under extreme hydro climatic conditions specific to the warm season in western Romania. The use of growth stimulus mixed with slaughterhouse sludge at optimal doses has determined also the increase of the seed quantities by up to 37% vs. in the absence thereof. In addition, the use of growth promoters to used fertilizers led to significant reductions of bioaccumulation of toxic metals in the case of Pb, reaching up to 62.1%. Residual bioaccumulation of lead in grass crops imposes the need to manage them under monitored regime but seeds harvested from inert land, treated with organic fertilizer may be used in future seeding.

It is necessary that all crops resulting from the fly ash and boiler slag deposits, fertilized with slaughterhouse sludge in this/absence of plant growth stimulus, to be closely monitored. Plant crops can be used in animal feeding only if the bioaccumulation of metals does not endanger their health.

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Article

TEACHING BIOINFORMATICS: ONLINE MOLECULAR DOCKING

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ABSTRACT

This study describes the use of molecular docking technique applied on MTiAutoDock online server in teaching bioinformatics for students in chemistry, biology and biochemistry. It also illustrates the use the online sever to predict the cytochrome P450 2C9 enzyme interaction with the drug ibuprofen. The benefits of using the online docking server for teaching molecular docking to the students in chemistry, biology and biochemistry and in a country where the powerful computing facilities are missing are also discussed.

Keywords: teaching, bioinformatics, MTiAutoDock.

1. INTRODUCTION

In the universities of Timisoara the students in chemistry, biology and biochemistry do not have solid informatics skills as there are not topics of basic programming and statistical knowledge in their university curricula. Also, with a very few exceptions, they have not any knowledge concerning Unix or Linux operating systems. Taking into consideration these features, the online bioinformatics tools are very useful and inexpensive teaching and learning instruments allowing both the teachers and students to perform bioinformatics analysis. Teachers have the opportunity to illustrate how the bioinformatics tools are applied and students have the possibility to perform computational tasks in their own rhythm, to repeat the tasks as many times as they need in order to have a detailed view of the process they analyze.

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Last decades are characterized by the development of the internet and increase of the on-line resources. Also, the bioinformatics topics are now present in the university curricula for biology, biochemistry and computer science, especially at the master degree level. In order to better prepare students for master degrees and research activities there is a real need to teach bioinformatics in the undergraduate classes of biology and biochemistry [1, 2] and even to the high school level [3, 4]. Starting to 2011, PLOS Computational Biology's Education section introduced a new collection "Bioinformatics: Starting Early" (http://www.ploscollections.org/article/browseIssue.action?issue=info:doi/10.1371/issue.pcol.v03.i09). This collection is dedicated to bioinformatics teaching in secondary schools and it proves the great interest in introducing bioinformatics early in the study programs of pupils and students.

Another characteristic of the last decades is the massive accumulation of data concerning the sequences and structures of the biological macromolecules. These data are now structured and deposited in data bases, most of them being free accessible for educational purposes. The bioinformatics training help the students to access these databases, to efficiently use the information and the proposed analysis tools [5]. All these illustrate the need of teaching bioinformatics at least at the university level.

On-line facilities for teaching and performing bioinformatics analysis increase continuously. Forming competences for using on-line facilities to the students in chemistry, biology and biochemistry corresponds to the actual trend in modern chemical and biological sciences teaching. Once the students become familiar to using the databases and a few basic bioinformatics tools, such as to visualize and analyze the spatial structures of the biological molecules and to simulate their interactions with different ligands through molecular docking technique, they are able to explore new on-line bioinformatics facilities. Usually, tutorials and illustrations are available online to help students to learn how to use every resource and gather data that may answer their initial questions or can be use in further studies.

Besides the great diversity of the bioinformatics tools, the molecular docking is one of the computational tools that help the students to understand the interaction between biologically relevant molecules (proteins, nucleic acids, carbohydrates, lipids) at the atomic level, as well as to clarify fundamental biochemical processes, especially those based on the protein-protein and protein–ligands interactions. There are many servers allowing online protein-protein (http://bioinformatictools.blogspot.ro/2011/12/protein-protein-docking-servers.html) and protein-ligand (http://bip.weizmann.ac.il/toolbox/structure/binding.htm) molecular docking**,** a comprehensive list of docking web services, databases and computer-aided drug design tools being found on the Click2Drug server (http://www.click2drug.org/)**.**

Molecular docking tools are an integral part of many actual structure-based drug discovery studies. From this point of view, it becomes important for students to be able to use docking programs for a particular study.

Studying science means that students learn to use the scientific methods, understand how to develop a scientific theory, evaluate different data, test hypotheses and make predictions. They must develop ways of thinking and acting in the practice of science and use the correct language and different scientific methodologies to collect, organize, interpret, calculate and communicate information. For example, to explain the correlations between the structure and function of biologically important macromolecules and their involvement into cell processes, the students must to understand first the specific characteristics of chemical substances and

macromolecules utilized by living systems. By using bioinformatics tools, students have the opportunity to broaden their competences in studying, understanding and communicating science.

This paper illustrates the use the MTiAutoDock online server [6] to perform molecular docking tasks in teaching bioinformatics for students in chemistry, biology and biochemistry at master level. The illustrated molecular docking computation concerns the prediction of the interactions of the cytochrome P450 2C9 (CYP2C9) enzyme with the drug ibuprofen.

2. METHOD

The topics of the bioinformatics course cover the followings: analysis of the protein's sequences and sequence alignments, analysis of the global and local structural properties of proteins (protein's backbone properties, surface properties such as electrostatics and hydrophobicity, surface roughness, surface pockets, cavities and protrusions) and prediction of the protein-ligand interactions based on molecular docking method. There are 28 hours of teaching and 28 hours of practical activities for the bioinformatics course. For every student, a distinct project concerning a bioinformatics study of the interaction between a protein and a specific ligand is asked for the final examination. Information concerning the protein's sequences is retrieved using the UniProt database [7] and the facilities therein (http://www.uniprot.org/). Information concerning the ligand structure and properties may be retrieved from ZINC database (http://zinc.docking.org/) [8]. Sequence alignment is illustrated using the Basic Local Alignment Search Tool [9] on-line (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and multiple sequences alignment is also performed on-line using CLUSTALW tool [10]. The structures of the proteins and protein-ligand complexes are retrieved from the Protein Data Bank (PDB) [11] and the interactive visualization and analysis of structures is performed using UCSF Chimera computational tool [12]. Fpocket on-line tool is used for identification and characterization of pockets and binding sites present on the protein surface [13]. The hydrophobicity distribution on the protein surface is visualized and analyzed using UCSF Chimera tool. Prediction of the protein-ligand interactions is performed using the molecular docking implemented under the on-line docking servers. In the followings, the use of 1-Click Docking server is illustrated.

Molecular docking is a computational technique that predicts the binding orientation of one small molecule at the binding site of a target macromolecule, usually a protein, and estimates the binding affinity [14]. Molecular docking studies use one of the two approaches: the geometry-based approach considering the surface complementarity of the ligand and protein [15] and the energetic-based approach computing ligand-protein pairwise interactions energies. There are both advantages and limitations for every approach.

MTiAutoDock is an online docking tool predicting the binding affinity of a single or multiple ligands to a protein based on the energetic approach and allows the download and/or visualization of the binding poses (http://bioserv.rpbs.univ-paris-diderot.fr/services/MTiOpenScreen/). There also is a video tutorial for using it (http://bioserv.rpbs.univ-paris-diderot.fr/services/MTiOpenScreen/#usage) that students must follow before to use the server.

3. RESULTS AND DISCUSSIONS

This part of our study illustrates the use of the MTiAutoDock online tool for predicting the binding affinity of ibuprofen, an analgesic and anti-inflammatory drug, to the CYP2C9 enzyme. CYP2C9 enzyme is known to metabolize approximately 15% of the clinically used drugs, such as antidiabetics, anticonvulsants, anti-inflammatories, anti-hypertensives, proton pomp inhibitors and anxiolytics [17]. The three dimensional structures both for the unbound protein and for its complex with the drug warfarine has been determined [18] and their codes entry in the PDB are 1OG2 and 1OG5 respectively. There also is a crystallographic structure of mutated CYP2C9 in complex with the drug flurbiprofen [19], PDB code entry 1R9O. In our study we use the structural file 1OG5 without the ligands and prepared for docking using Chimera computational tool [12].

The structural file for the drug ibuprofen is extracted from ZINC database [8]. The two files are uploaded of the web page of MTiAutoDock tool (figure 1).

Figure 1: Upload the structural files on MTiAutoDock web page

It is possible to perform a blind docking or to specify the binding site by its residues or by the coordinates of a selected atom belonging to the binding site. Also, the user may upload a ligand or may try one of the ligands that are found in a predefined compound library. The ligand is taken randomly or selected by the physico-chemical criteria defined by the user.

After running the docking, the poses can be visualized or download for further analysis (figure 2) and the binding energy is also delivered (figure 3). The best binding mode of ibuprofen to the CYP2C9 has a binding affinity of -8.32 kcal/mol. Considering the crystallographic structures of CYP2C9 in complex with flurbiprofen and warfarin, we also perform molecular docking predictions for these drugs binding to the enzyme as a control.

The use of MTiAutoDock tool to analyze the binding of flurbiprofen to CYP2C9 predicts the binding energy of -9.20 kcal/mol and the predicted binding energy of warfarin to CYP2C9 is -9.63 kcal/mol. These results suggest that ibuprofen binds to CYP2C9, but the interactions with flurbiprofen and warfarin are stronger

Figure 2: The best pose visualization for the binding of ibuprofen to CYP2C9

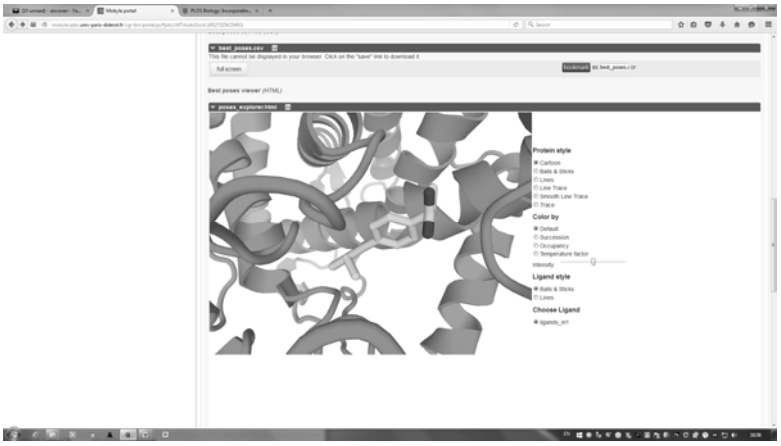
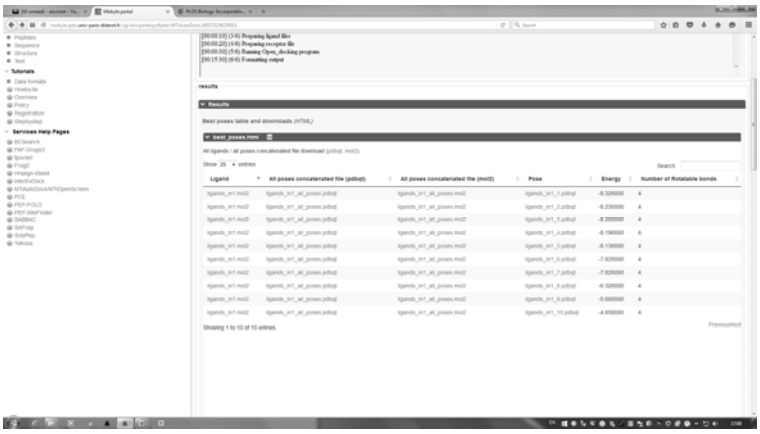


Figure 3: The interaction energies for all predicted poses for the binding of ibuprofen to CYP2C9



As the crystal structures of CYP2C9 in complex with the anti-inflammatory drug flurbiprofen and to the anticoagulant drug warfarin are solved, the residues identified as interacting to ibuprofen may be compared to those interacting to warfarin and flurbiprofen and it informs about the structural basis of drug binding to CYP2C9 .

4. CONCLUSION

In our faculty, the topic of bioinformatics is present in the study program of the master students in chemistry, biology and biochemistry only since 4 years. During this period, about 80 students have benefited from mentoring to use online tools to study biological processes at the molecular level. At the end of the semester students complete anonymous questionnaires about their personal benefits obtained by completing the course. Most students appreciate the totally new information and competencies regarding the use of bioinformatics methods to study chemistry, biology and biochemistry, especially the use of the biological databases and

of the bioinformatics tools for analyzing the biological interactions at the molecular level. They also consider that a bioinformatics course at the license level would be beneficial allowing them a better understanding of the structural levels of biological macromolecules and of their structure-function relationships.

The results presented in this study illustrates that online molecular docking facilities can be successfully employed as effective teaching tools to demonstrate the use of molecular docking to beginners in the field.

Free available on-line bioinformatics tools may be accessed by students from home, they only need their computers to be connected to the internet and they have the opportunity to repeat the steps they performed in classroom as many time as they need and in their own rhythm in order to obtain a complete understanding of both the bases of the bioinformatics tool they use and the explored biochemical processes. This opportunity contributes to a better understanding of the applications of bioinformatics tool and also may increase the interest of students to explore other tools and to perform other kinds of bioinformatics research without any cost.

The on-line teaching bioinformatics resources are important for both teachers and students because they develop interest to the students by facilitating understanding advanced studies and they promote quality human resource to undertake challenging research in the field of modern chemistry, biology and biochemistry. They also contribute to the interdisciplinary approach of the study of the physiological processes at the molecular level.

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Article

COMPARATIVE STUDY CONCERNING THE REMOVAL OF PHENOL FROM WASTEWATER ON SLUDGE AND ZEOLITE*

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ABSTRACT

The present study presents two adsorbents which have been comparatively used in order to remove phenol from wastewater. Sludge from the wastewater treatment plant of Timisoara and zeolite of commercial origin are cheap and easily available materials. The adsorbent materials were characterized using the EDAX technique. The influence of the contact time and of the adsorbent mass was watched over the adsorption equilibrium and the optimal working conditions were settled regarding the phenol adsorption on these two materials.

Keywords: phenol, sludge, zeolite, adsorption

1. INTRODUCTION

Nowadays, a broad range of industries take benefit of phenol and its derivates as raw materials, essentially serving for the production of different materials from plastics, epoxy resins or pesticides to pharmaceuticals [1]. On the other hand, when generated by the industries phenol and its derivates are significant contaminants of wastewater [2-6].

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To living organisms phenol and its derivates are highly toxic, harmful and corrosive being rapidly absorbed through the skin or following inhalation and ingestion. Once absorbed, the phenol and its derivatives are distributed throughout the body, concentrating mainly in the liver and kidneys.

In human body, phenol follows several stages of metabolism: conjugation, oxidation and excretion. The main mechanism consists of conjugation reactions (direct sulfation and glucuronidation) that result in the formation of glucuronide and sulfate ester. The phenol which is not directly conjugated may be the substrate for an oxidative metabolism, when its oxidation at dihydrodiols compounds occurs. These, in turn, can participate in conjugation reactions with the glucuronic acid and the sulfate. Through an enzymatic mechanism, the phenol and quinones such as reactive intermediates, in turn, are substrates for conjugation [7,8].

Regardless of the route of access into the body, phenol does not significantly accumulate in the body, being rapidly eliminated. The elimination occurs mostly through urine as sulfate and glucuronide conjugates. Therefore, direct conjugation of phenol and glucuronic acid or phenol and sulfate are the main mechanisms of detoxification, which represent the greatest part of phenol metabolism. [7].

According to the product Safety Data Sheet provided by some of the largest phenol producers such as SLOVNAFT VÚRUP [9], SIGMA-ALDRICH [10], AMRESCO [11], LIFE TECHNOLOGIES [12], AXIALL, LLC [13], SCIENCELAB.COM, INC. [14], CARLO ERBA REAGENTS [15], phenol presents:

- ✓ *acute dermal toxicity* (it is toxic in contact with skin, corrodes / irritate the skin; it causes severe skin burns, eye damage, even corneal perforation);
- ✓ *acute inhalation toxicity* (it is toxic if inhaled as vapour, it can lead to total intoxication damaging the CNS);
- ✓ *acute oral toxicity* (it is toxic if swallowed; it can lead to painful cauterization of the digestive tract and subsequent symptoms of damage to the CNS and can have lethal effects*);*
- ✓ *systemic toxicity over a target organ*- central nervous system, blood, liver, kidney (it can cause damage to organs through prolonged or repeated exposure);
- ✓ *susceptibility of causing genetic anomaly* (it is suspected of mutagenicity);
- ✓ *susceptibility of causing cancer*.

The particular high toxicity of phenol imposes a series of measures regarding its removal from various matrices. There are several methods for the removal of phenol from aqueous solutions, such as distillation, extraction, membrane processes, adsorption, chemical precipitation, ion exchange, reverse osmosis, chemical oxidation, solvent extraction / stripping gas, complexation, bioremediation.

Adsorption is an effective separation process, intensely studied and recently used for the treatment of industrial effluents and domestic wastewater.

A suitable adsorbent for wastewater treatment must possess a number of qualities [16]:

- ✓ to present a high affinity for the target compounds, translated into a high adsorption capacity;
- ✓ to ensure the greatest possible degree of removal from the wastewater of the targeted pollutant vector;
- ✓ to be stable and renewable;
- ✓ to allow desorption and recovery of the adsorbed compound;
- ✓ to have a low cost;
- ✓ to be tolerated by the environment.

Even though many adsorbents have been tested and reported in the literature, rarely they posses all of the characteristics mentioned above. This is the case for: activated carbon obtained in various ways [6, 17-22], activated carbon with various additions [23], ash and modified ash [24-29], hydroxyapatite [5, 30], polymeric resins or polymeric composites [31-33], alumina, silica gel, zeolite [34-37], chitin-chitosan [38-41], chitosan-alginate [42], modified bentonite and kaolinite [43], iron oxide [44]. Among them, natural and modified zeolites are of great importance [45-53].

For the present study two adsorbent materials have been used, sludge from Timisoara water treatment plant and zeolite of commercial origin, for the removal of phenol from wastewater. The aim was the comparative assessment of the performance of these two materials, which are quite affordable and can be easily procured.

2. METHODS AND MATERIALS

Adsorbent materials

The sludge used for the adsorption study and phenol removal from aqueous solutions originates from the wastewater treatment plant "Stan Vidrighin" of Timisoara.

The sludge was dried in an oven at 95°C for 12 hours, after which it was ground and sieved through a metallic sieve.

The zeolite used as adsorbent material comes from the CEO EcoNatura Company, Romania. Mineralized zeolitic volcanic tuff has been obtained from volcanic rocks rich in natural zeolite, in which clinoptilolite is predominant.

Reagents and Solutions

The following reagents were used: phenol (Fluka), 4-amino-antipyrine (Sigma-Aldrich), hexacyanoferrate (III) (Fluka), 25% ammonia (Sigma-Aldrich), ammonium chloride (Sigma-Aldrich), chloroform (Sigma-Aldrich), double distilled water.

All reagents were of analytical purity.

The stock solution of phenol, with a concentration of 1 g/L, was obtained by dissolving the appropriate amount of phenol, weighed to an accuracy of ± 0.0001 mg, in double distilled water, in a volumetric flask.

Phenol standard solutions, of various concentrations, were prepared by serial dilutions of the stock solution with double distilled water.

Instruments and devices

For the adsorption experiments, it was used a thermostated shaker type Vibramax 100 Heidolph whose stirring rate was set at a constant value of 200 rpm.

The phenol concentration from the initial sample and from the one subjected to the absorption was determined spectrophotometrically according to the method ISO 6439:2001 with the aid of a T90 UV / VIS dual beam spectrophotometer type, PG Instruments Ltd. The working wavelength was 460 nm, using a 1cm quartz cuvette.

Working methodology

The measurements were carried out at room temperature (25 ± 1°C), without pH adjustment of the solutions.

For each adsorption experiment, the appropriate amount of adsorbent material was added to 50.0 mL of phenol solution in a 100 mL beaker. The samples were shook at a constant speed (300 rpm) for 15, 30, 45, 60, 90, 120 and 150 minutes.

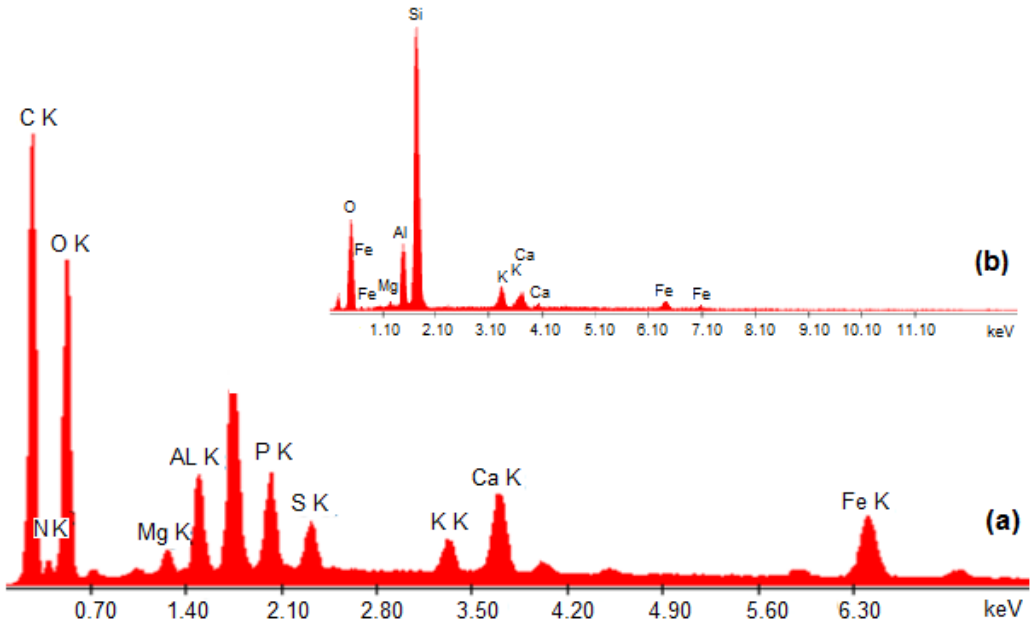
After the time in contact, samples were filtered with a vacuum pump and the filtrate was used for the spectrophotometric determination of the residual phenol.

3. RESULTS AND DISCUSSIONS

Characterization of the adsorbent materials

The morphology of the adsorbents surface was characterized by energy dispersive X-ray analysis (EDAX). EDAX studies of the dry sludge and zeolite samples were performed using an electron microscope FEI Inspect S.

Figure 1: EDAX spectra for (a) sludge and (b) zeolite



In Figure 1 (a) and (b) are given the EDAX spectra for dried sludge and zeolite. Table 1 presents the elemental composition of the dried sludge.

In the case of dry sludge, EDAX spectra indicate the presence of metals such as Al - 2.50%, Fe - 2.00% Ca - 1.24%, Mg - 0.60%, K - 0.46%, along with other elements such as N - 6.09% Si - 3.90%, P - 1.52% and S - 0.608%. As expected, best represented elements are C - 51.06% and O - 30.39%, which indicate the presence of organic matter and water.

For comparison, the EDAX data from Figure 1 (b) are presented revealing the main elements in zeolite: Si, Al and O, as confirmation for zeolite as an aluminosilicate. Beside these elements, zeolite contains much smaller amounts of K, Ca, Mg and Fe. The EDAX data also indicate that the Si/Al ratio is approximately 4.5. In the specialized literature Si/Al

reports are indicated ranging from 4.0 to 5.3 corresponding to chemical composition of pure clinoptilolite. On the other hand, heulandite, another zeolitic mineral with a similar structure of clinoptilolite has a Si/Al ratio of 2.7 [16, 54, 55]. EDAX data therefore prove that zeolite used for this specific study as adsorbent material has the basic structure of clinoptilolite type.

Table 1: Elemental composition of dry sludge

EDAX ZAF Quantification (Standardless)
Element Normalized
SEC Table : Default

Element	Wt %	At %	K-Ratio	Z	A	F
C K	51.06	60.95	0.1706	1.0136	0.3296	1.0003
N K	6.09	6.24	0.0064	1.0061	0.1041	1.0006
O K	30.39	27.24	0.0431	0.9994	0.1420	1.0001
MgK	0.60	0.36	0.0019	0.9637	0.3332	1.0021
AlK	2.05	1.09	0.0088	0.9364	0.4565	1.0029
SiK	3.90	1.99	0.0215	0.9648	0.5698	1.0018
P K	1.52	0.70	0.0090	0.9338	0.6334	1.0014
S K	0.68	0.30	0.0048	0.9590	0.7346	1.0014
K K	0.46	0.17	0.0042	0.9098	0.9852	1.0050
CaK	1.24	0.45	0.0118	0.9312	1.0191	1.0022
FeK	2.00	0.51	0.0184	0.8635	1.0674	1.0000
Total	100.00	100.00				

Optimal conditions of work. Influence of contact time

In order to study the influence of the contact time, samples of a given concentration of phenol were stirred together with different amounts of adsorbent at certain periods of time: 15, 30, 60, 90, 120 and 150 minutes in case of zeolite and 15, 20, 30, 60, 90 and 120 minutes in case of sludge. The yield variation and the adsorption capacity variation with contact time have been observed. Reaching the plateau region corresponds to the establishment of the adsorption equilibrium.

The plots η [%] = f (t) and q_e = f (t) for the adsorption of phenol on zeolite, are given in Figure 2 (a) and (b), and for the adsorption of phenol on sludge in Figure 3 (a) and (b).

When adsorption is performed onto sludge, Figure 2 (a) and (b) shows that, for the initial concentration of phenol of 1 mg/L (1 ppm), the highest adsorption occurs within the first 30 minutes, after which the adsorption yields and the adsorption capacity increase further tending towards an equilibrium that is reached after 60 minutes.

In this phase, we can assert that the removal of phenol from solution ceases. Only one exception: for 0.50 g of sludge the adsorption equilibrium is reached after 90 minutes. Therefore we chose the *optimal contact time on sludge of 90 minutes*.

Depending on the amount of sludge, adsorption yield is between 39.35% (on 0.50 g sludge) and 65.44% (on 3.00 g sludge).

The adsorption onto zeolite reveals the plateau, for the same initial concentration of phenol of 1 mg/L (1 ppm), after 90 minutes (Figure 3 (a) and (b)). In this case, the exception comes for 0.50 g of zeolite, where the adsorption equilibrium is reached after 120 minutes of contact. To be on the safe side, the *optimal contact time on zeolite* was chosen at *120 minutes*.

The range in which adsorption yield is comprised also depends on the amount of adsorbent: between 42.13% (per 0.50 g zeolite) and 66.12% (by 3.00 g zeolite).

Figure 2: (a) η [%] = f(t) and (b) q_e [mg/g] = f(t), at phenol adsorption on sludge; $C_{i\ Ph}$ = 1 mg/L (1 ppm)

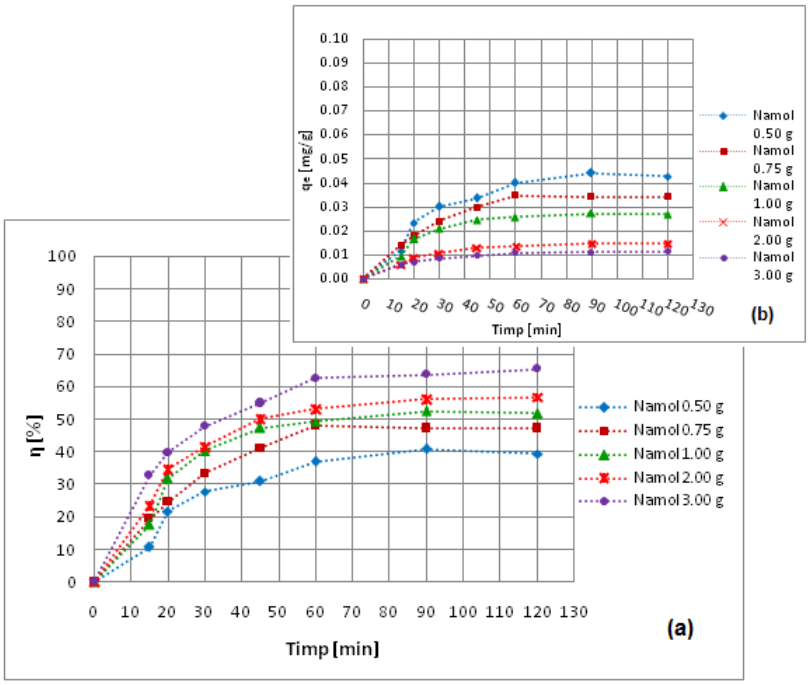
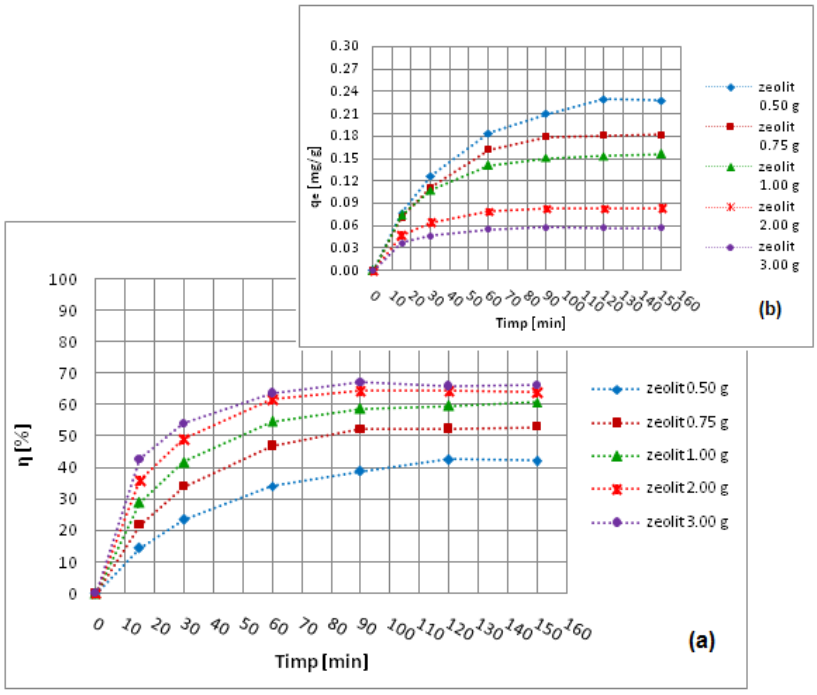


Figure 3: (a) η [%] = f(t) and (b) q_e [mg/g] = f(t), at phenol adsorption on zeolite; $C_{i\ Ph}$ = 1 mg/L (1 ppm)



Optimal conditions of work. The influence of adsorbent mass

To determine the influence the mass of adsorbent brings, the phenol samples were placed in contact with 0.50; 0.75; 1.00; 2.00 and 3.00 g of each adsorbent, between 15 and 150 minutes. The variation of the yield and of the adsorption capacity with adsorbent mass has been recorded. The obtained plateau region corresponds to the establishment of the adsorption equilibrium.

The plots η [%] = f (m_{ads}) and q_e = f (m_{ads}) for the adsorption of phenol on both adsorbents are given in Figures 4 (a) and (b) and 5 (a) and (b).

In case of sludge (Figure 4 (a) and 4(b)) the adsorption equilibrium is reached for an amount greater than 2.00 g of adsorbent. In this case, it was considered that for a phenol solution of 1 ppm, the optimum mass of adsorbent is 2.00 g.

Figure 5 (a) and (b) indicate that phenol adsorption onto zeolite reaches the equilibrium also for quantities larger than 2.00 g of adsorbent. This value was considered the optimum mass of zeolite for 1 ppm phenol adsorption.

Figure 4: (a) q_e [mg/g] = f(t) and (b) η [%] = f(t), at phenol adsorption onto sludge; $C_{i\,Ph}$ = 1 mg/L (1 ppm)

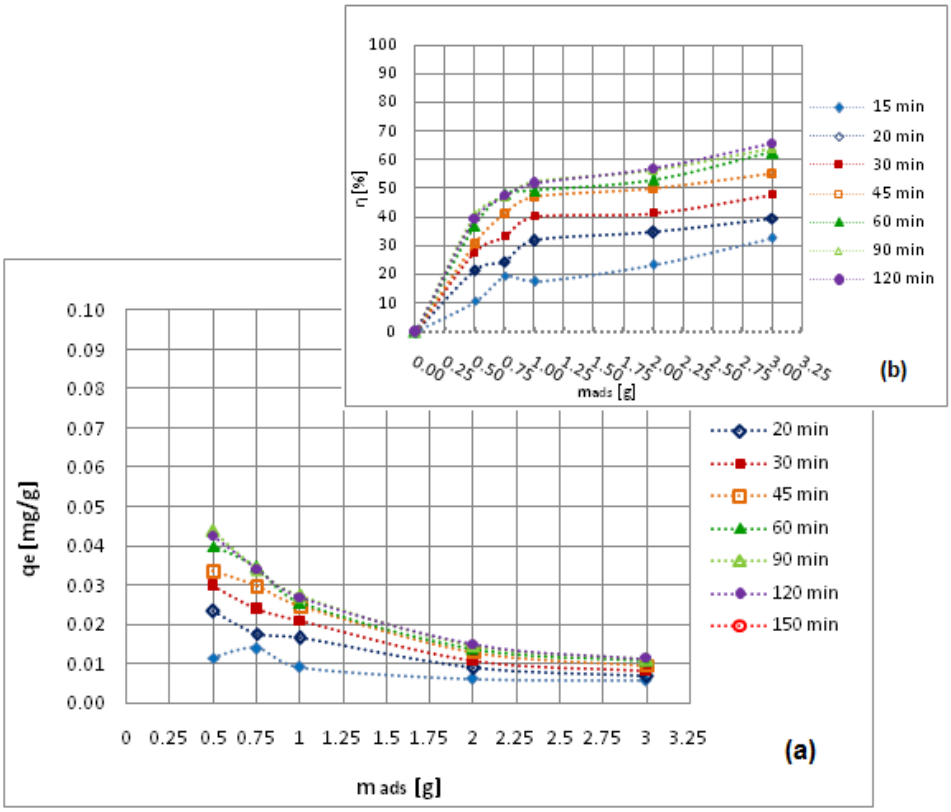
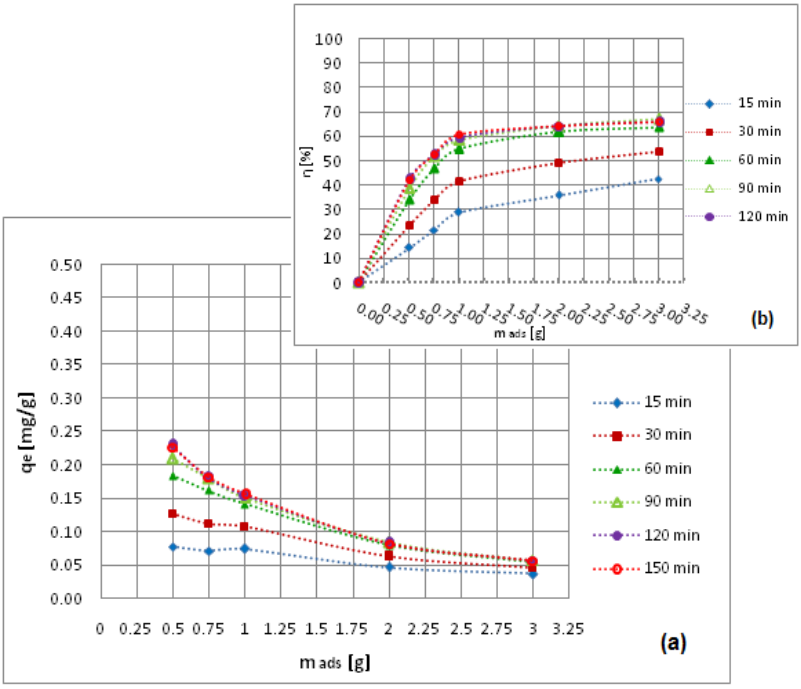


Figure 5: (a) q_e [mg/g] = f(t) and (b) η [%] = f(t), at phenol adsorption onto zeolite; $C_{i\ Ph}$ = 1 mg/L (1 ppm)



4. CONCLUSION(S)

There have been studied and established optimal working conditions for the phenol adsorption from solutions with an initial concentration of 1 ppm, on zeolite and on sludge.

It has been found that, at room temperature, the most favorable working conditions for the adsorption of phenol from a solution with a concentration of 1 mg / L, are:

- on zeolite: 120 minutes contact time and 2.00 g adsorbent / 50 mL sample;
- on sludge: 90 minutes contact time and 2.00 g adsorbent / 50 ml sample.

In terms of contact time, the sludge is more indicated as adsorption material, having a faster adsorption time.

In terms of performance, under optimal working conditions (the ones mentioned above), the yield of adsorption is slightly better on zeolite than on sludge. But not also the adsorption capacity, which is net superior in case of the zeolite:

- zeolite: 64.28% adsorption efficiency and 0.083 mg /g adsorption capacity;
- sludge: 56.27% adsorption efficiency and 0.015 mg /g adsorption capacity.

The studies have shown an increase of the adsorption efficiency of phenol, as well as the contact time increased, as also at a weight increase of the adsorbent. This was due to the fact that the number of adsorption sites increased, at the increase of the adsorbent dose [5, 16]. A further increase of the contact time or of the adsorbent dosage over the optimal value is unjustified, the increase of these parameters becoming in this case insignificant, suggesting an evolution towards a plateau, a balance zone. The explanation is, that in time, the amount of

active sites decreases, the adsorbent becoming more agglomerate, thus hindering the free movement of its inner particles [4, 16].

On the other hand, the value of the adsorption capacity is high at low doses and reduced at high doses of adsorbent. Therefore, in accordance with the literature data [5, 56], has been noticed a decrease of the adsorption capacity, at an increase of the adsorbent quantity. This seems obvious, if we consider the mood of defining and of calculus, of the adsorption capacity. At the increase of the adsorbent mass, the quantity absorbed per its unit mass remains constant and the number of active sites remained the same. The number of mass units grows faster, in comparison to the amount adsorbed on them, which has as result a decrease of the adsorption capacity value. Transposed on the adsorption mechanism, the explanation of this conduct must consider several factors. The Langmuir approach of the adsorption, according to which the saturation is reached at the adsorbent surface coating with a monolayer of adsorbed molecules, may explain why the amount adsorbed per mass unit does not increase proportionally to the one absorbed on the entire absorbent mass. However, the finding that the increase of adsorption is not commensurable with an increase of the adsorbent dose, suggests that there are a number of adsorption sites, which remain unsaturated during the adsorption, which leads to a lower use of the adsorption capacity of the adsorbent [5, 16].

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Article

ANTIMICROBIAL AND CYTOTOXIC POTENTIAL OF SILVER NANOPARTICLES SYNTHESIZED USING *RHEUM EMODI* ROOTS EXTRACT

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ABSTRACT

Antimicrobial and cytotoxic potential of silver nano particles (AgNPs) synthesized through greener route using *R. emodi* root extract was investigated. The synthesis of AgNps was confirmed by visualizing the colour change which shows surface plasmon resonance (SPR) peak at 425 nm. FTIR analysis indicates the involvement of anthraquinone pigments, in capping and stabilization of AgNps. FESEM, XRD, TEM, with EDX display the formation of crystalline AgNps with face centered cubic (FCC) symmetry with average size 27.5nm. AgNps were found to exhibit significant antimicrobial activity. Similarly, synthesized AgNps show substantial change in cytotoxicity against human breast lines (MCF-7 cell lines).

Keywords: *Rheum emodi*, Green synthesis, Silver nanoparticles, Antimicrobial, Cytotoxic potential.

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1. INTRODUCTION

In the present era of nanotechnology, nanoparticles have found tremendous applications in various fields such as optical polarizability, catalysis, electronics and medicine [1-3]. Copper, silver, gold have unique properties that offer numerous application in audiophile, photonics, photography, biological labelling and also have scientific application [4-5]. Literatures have reported the use of diverse biological regime such as plants extract, algae, diatom, fungi and bacteria for the synthesis of nanoparticles [6-10]. But, the biogenic synthesis of these nanoparticles using plant biomass has been found to be more viable owing to its simplistic methodology and easy to scale up processes. This increases the scope of investigation and regulation of nanoparticles at cellular stage, drug delivery, hepatitis B, cancer detection and treatment, environmental pollution control, ceramic coatings, HIV/AIDS treatment, textile industries etc. [11-16].

Among diverse nanoparticles, silver nanoparticle has been found to be more promising due to its anti-inflammatory, anti-angiogenesis, antifungal, antiviral, anti-inflammatory, antibacterial, anti-platelet activity, diagnosis and against cancer cells which makes them vital [17-21]. It has gained attention over the years because of distinctive properties, such as good conductivity, low sintering temperature, chemical stability, optical properties and antimicrobial coating [22-25].

Rheum emodi is one of the natural sources which belong to polygonaceae family. It is an important medicinal plant and the main part used as drug is root. The plant contains several biomolecules such as flavonoids, saponins especially anthraquinones. The anthraquinone derivatives such as Aloe emodin, Emodin, Physcion, Rhein and Chrysophanol etc. show remarkable antimicrobial, antiviral, antifungal, antioxidant, anti-Parkinson's and anticancer activities [26-27].

Only few studies have been reported on root mediated synthesis of biogenic metal nanoparticles using various plant resources such as *Rumex hymenosepalus* [27], *Erythrina indica* [28], *Delphinium denudatum* [29], *Nelumbo nucifera* [30], *Streptomyces hygroscopicus* [31], *Momordica charantia* [32], *Acacia nilotica*[33]. The present study focusses on biogenic synthesis of silver nanoparticles using methanol root extract of *R. emodi* evaluated for its antimicrobial, antifungal and anticancer applications.

Figure 1: (a) UV-vis spectra of *R.emodi* root crude extract and Synthesized Ag NPs. (b) XRD pattern of Synthesized Ag NPs. FTIR transmittance of (c) *R.emodi* root crude extract and (d) Synthesized Ag NPs.

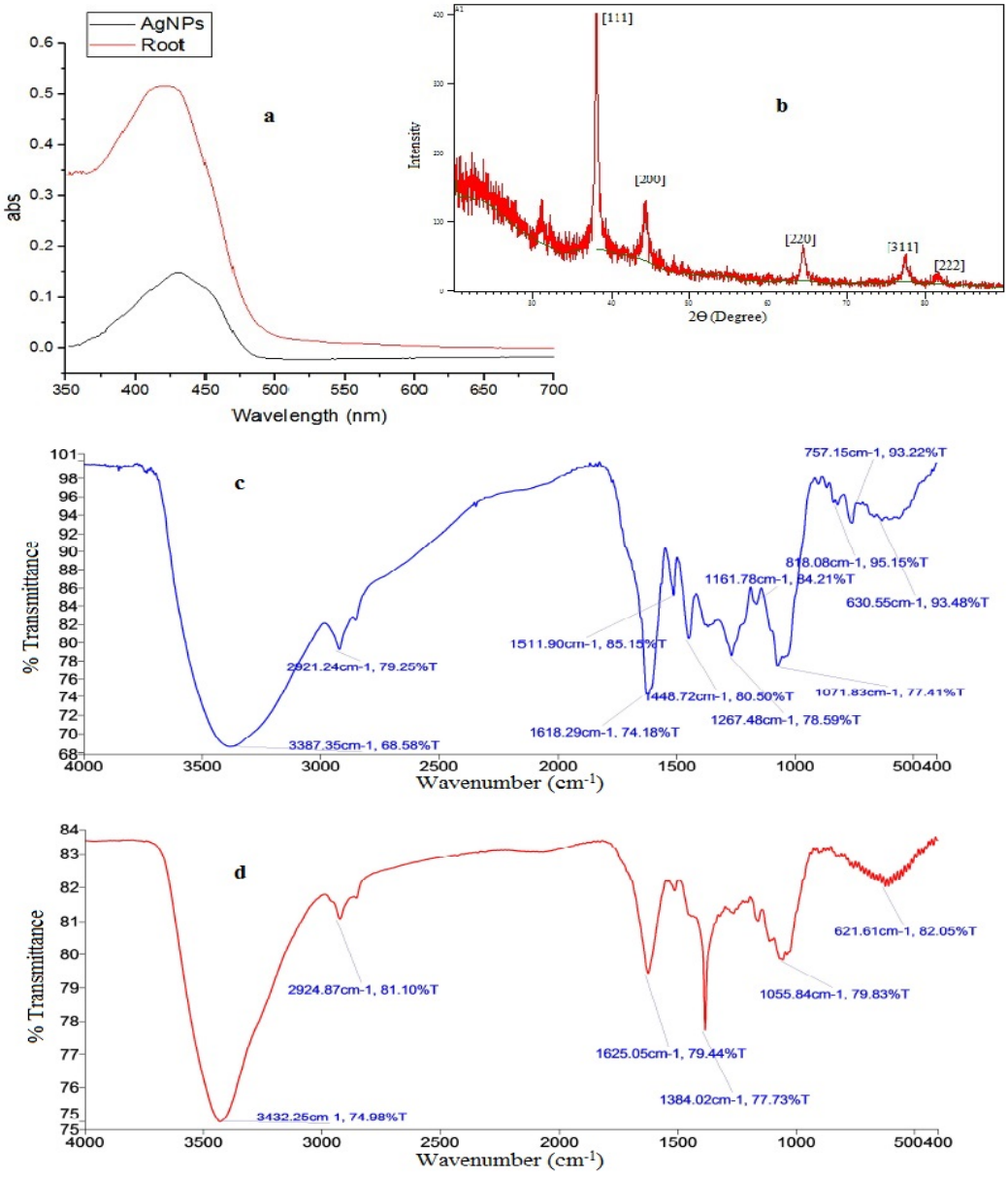

Figure 2: (a-b) TEM micrograph of synthesized Ag NPs, (c-d) FESEM micrograph of synthesized Ag NPs showing spherical shape and (e) EDX shows strong metal peaks of silver

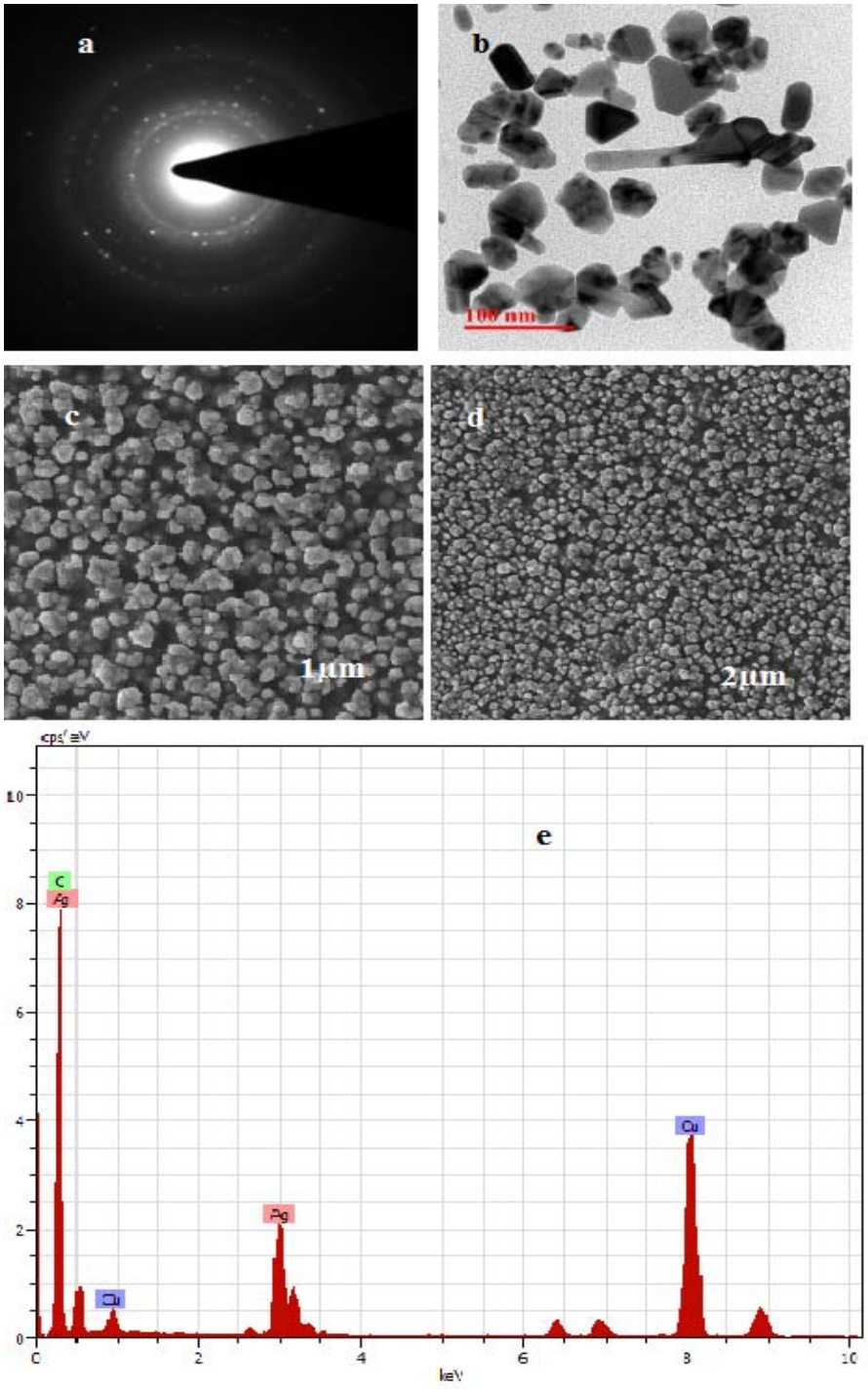

Figure 3: Antimicrobial and antifungal activity of synthesized Ag NPs using *R.emodi* root crude extract (A1) and *R.emodi* root crude extract (A) against different human pathogens and fungi's (a) *Streptomyces griseous*, (b) *Bacillus subtilis* (c) *Staphylococcus aureus* (d) *E.coli,*(a1) *Aspergillus niger*, (b1) *Fusarium oxysporum*. Concentration range 50 and 100mg/ml.

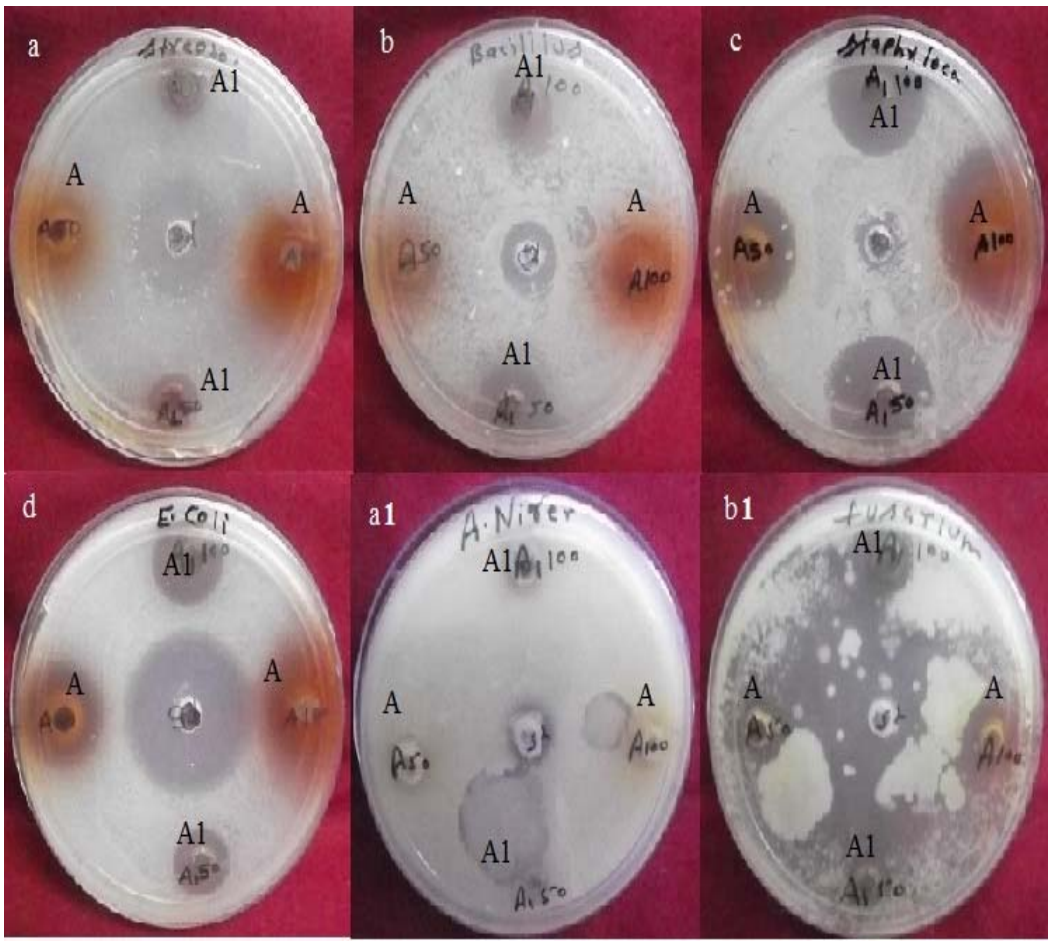

Figure 4: Anticancer activity of Ag NPs and crude extract (a) Control (b) Positive control (c) *R.emodi* root crude extract – 250 µg (d) Synthesized AgNP's – 250 µg. Effect of cell viability 0020and cytotoxicity of (e) *R.emodi* root crude extract and (f) synthesized Ag NPs of *R.emodi* root crude extract in MCF-7 cancer cells

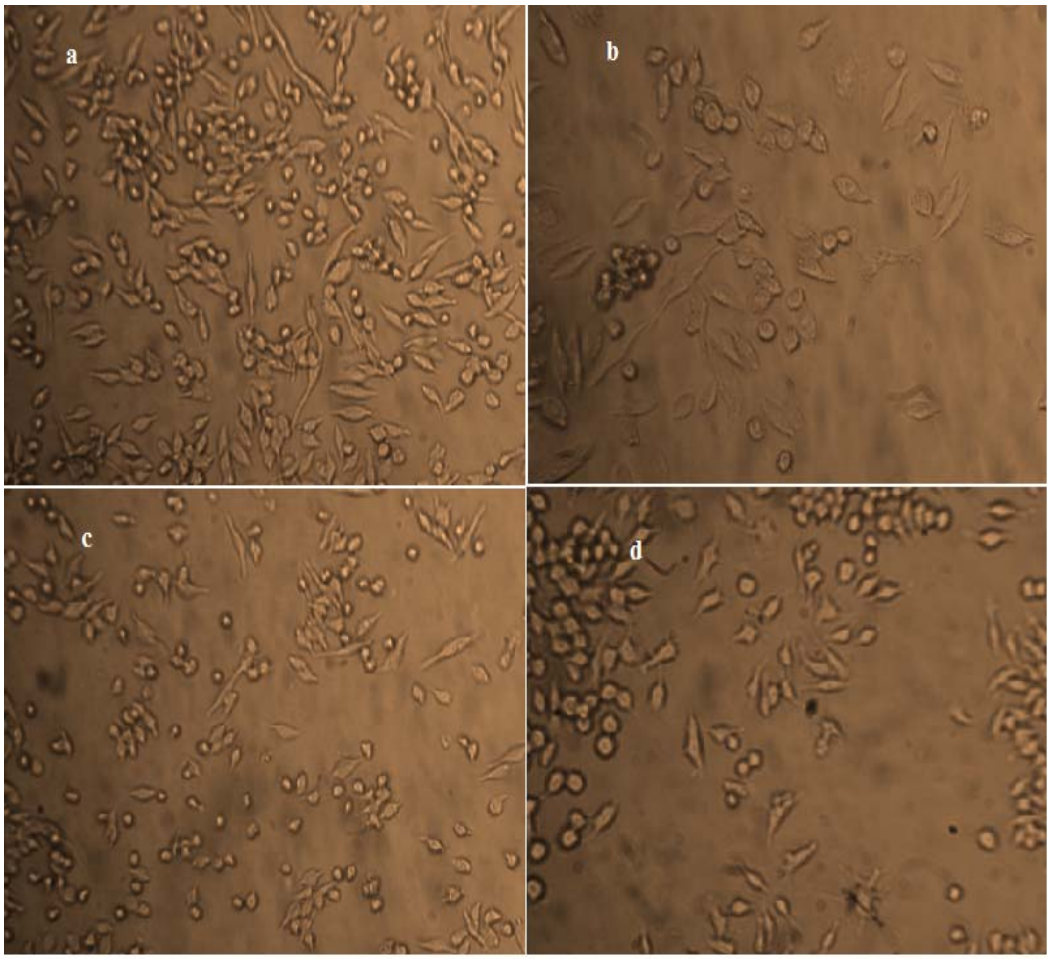

Table 1: Inhibitory action of synthesized Ag NPs and *R.emodi* crude root extract against human pathogenic bacteria & fungi

Test strain	Concentration of crude extract 50mg/ml , 100mg/ml		Concentration of Ag NPs 50mg/ml, 100mg/ml	
	Zone of inhibition in mm		Zone of inhibition in mm	
Bacillus subtilis	9 ±0.5	12 ±0.5	18±0.5	22 ±0.5
Staphylococcus aureus	28 ±0.5	38 ±0.5	32±0.5	34 ±0.5
Streptomyces griseous	9 ±0.5	12 ±0.5	14 ±0.5	16 ±0.5
Escherichia coli	18 ±0.5	22 ±0.5	20 ±0.5	30 ±0.5
Aspergillus niger	Nil	12 ±0.5	Nil	14 ±0.5
Fusarium oxysporum	16 ±0.5	22 ±0.5	13 ±0.5	20 ±0.5

Table 2: Percentage of cell viability and cytotoxicity of *R.emodi* root crude extract and synthesized AgNPs

Test	Root extract Concentration (µg/ml)					Ag NPs Concentration (µg/ml)					PC	C
	50	100	150	200	250	50	100	150	200	250		
% of Viability	83.78	74.94	65.35	55.73	46.57	77.32	64.76	53.70	42.40	34.13	26.11	100
% of cytotoxicity	16.21	25.05	34.64	44.26	55.43	22.67	35.23	46.29	57.59	65.87	73.89	0

2. METHOD

2A. Chemicals

Silver nitrate (AgNO3), A.R. and methanol, A.R. used in this study were procured from Merck (India) All other analytical reagent were purchased from Merck (Germany).

2B. Extraction of root extract and Synthesis of AgNPs

Roots of *R. emodi* were shade dried and pulverized in Wiley mill to powder form. The air dried and crushed root (250g) of *R. emodi* was extracted with methanol solvent in soxhlet extractor and the extract was concentrated under the reduced pressure. The same methanolic extract was used as a crude extract for further analysis. For the synthesis of AgNps; 10ml of root extract were dissolved in 500ml of double distill water and then boiled for 7-8minutes before decantation. The 10ml of obtained supernatant was mixed with 50ml of 1mM $AgNO_3$. The reaction mixture was incubated until colour changes from dark brown to orange at the room temperature.

2C. Characterization of AgNPs

The reduction of silver ions was monitored by UV-Visible (LAMBDA 750 Perkin Elmer) spectrophotometer at different time intervals (0h, 12h, 24h) between 300-700nm wavelength range. FTIR (Compact Perkin Elmer) spectral analysis in the range between 4000 cm^{-1} and 400 cm^{-1} was carried out to identify the possible biomolecules present in the *R. emodi* root extract which are responsible for reduction and stabilization of AgNPs. The shape and size of AgNPs were described by Transmission electron microscopy (TEM; TecnaiG^2 T20 ST) with EDX equipment. EDX analysis showed a strong elemental silver peak and morphological measurements which were observed by Field emission electron microscope (FESEM; Nano Nova 450). X-ray diffractometer (Panalytical X-Pert Pro) analysis describes crystallinity and size of synthesized AgNPs. [29]

2D. Antimicrobial Assay

i) Antibacterial activity

The antibacterial activity of root extract and AgNPs against Gram +ve (*Bacillus subtilis, Streptomyces griseous* and *Staphylococcus aureus*) and Gram –ve (*Escherichia coli*) human pathogens were studied by agar well diffusion method. All pathogenic microorganisms were grown at 37ºC for overnight in Mueller- Hinton agar (Hi Media India) broth. Wells Mueller-Hinton agar plates were prepared and overnight grown bacterial suspension (100µl) was swapped uniformly on the surface of a Mueller-Hinton agar plates. After 24h incubation at 37ºC, zone of inhibition were measured [31].

ii) Antifungal activity

Anti-fungal activity of the root extract and AgNPs were investigated by agar well diffusion method. The fungi were grown on Sabouraud's Dextrose Agar (SDA) for 72h at 37ºC. Fungi were spread on Sabouraud's Dextrose Agar (SDA) plates. Four discs were placed on each agar plates. *R. emodi* root crude extract and AgNPs (50 and 100mg/ml) were kept in each discs of each Petri plates with respective pathogens. After incubation of 72 h, diameter of zone of inhibition around each disc and the results were recorded [16, 32].

2E. Cytotoxicity Potential

i) Cell culture
Breast cancer cell lines (MCF-7) were maintained in MEM medium, supplemented with 10% Fetal Bovine Serum (FBS), 1% glutamine at 37ºC with 5% CO_2.

ii) Measurement of cytotoxicity by MTT assay
Cytotoxicity effect of synthesized AgNPs and root crude extract were performed on breast cancer cell lines (MCF-7 cells) measured using MTT assay. Briefly cultured MCF-7 cells (1.2×10^4 cells/ml) were plated on 96 flat-bottom well plates, then cells were exposed to different concentration of synthesized AgNPs and root crude extract (50, 100, 150, 200 and 250µg/ml) respectively and allowed for 24 h incubation at 37º C in 5% CO_2 atmosphere. After incubation, 10µl of MTT (5mg/ml) was added in each sample separately. After 4 hours of further incubation at 37 º C and 5% CO_2 atmosphere, 100 µl of DMSO was added to dissolve formazan crystals. Then, the absorbance was measured at 570 nm with reference filter as 655 nm in a microtitre plate reader. Cyclophosphamide was used as a positive control.

3. RESULTS AND DISCUSSIONS

3A. Synthesis of Ag NPs
Methanol extract of *R emodi* roots was used for the synthesis of silver nanoparticles which act as a reducing agent. The formation of AgNps was visibly characterized due to change of colour from dark brown to orange. After addition of root extract, the complete reduction of silver ions was achieved by 24 hours of incubation at room temperature because of the excitation of surface plasmon resonance of AgNps. The solution was observed extremely stable even after month of reaction, with no evidence of particle aggregation [41, 35].

The phytochemical analysis of *R emodi* roots revealed the presence of physcion, chrysophanol emodin, aloe emodin, rhein anthraquinones[26, 34]. These anthraquinones may be responsible for the formation of AgNps by reducing $AgNO_3$ and acting as a capping agent to provide stability to the medium and prevent agglomeration.

3B. Characterization of silver nanoparticles

i) UV-Visible Spectroscopic Analysis: The UV-Visible spectrum (Fig. 1: a) of synthesized AgNps showed intense peak at 425nm. It was due to excitation of longitudinal plasmon vibration in AgNps solution. The increase in intensity of silver nitrate solution with respect to crude root extract has indicated the formation of increased number of silver nanoparticles in solution [35, 36].

ii) XRD Analysis: The crystalline nature of synthesized AgNps was determined by XRD analysis (Fig.1: b). The diffraction pattern was recorded in the scanning mode at 40 KV and

30mA, with Cu Kα radiation. The XRD pattern indicated peaks at 2θ values of 38.17^0, 44.33^0, 64.44^0, 77.34^0 and 81.33^0 corresponding to the face centered cube(FCC) plane(111, 200, 220, 311) of silver crystals respectively which represents crystalline nature of AgNps. The presence of unpredicted peak at 31.84^0 may be due to anthraquinones belong to the *R. emodi* root extract. The mean particle diameter of the synthesized AgNps was found to be 27.5 nm using Debye-Scherrer equation

$$D = \frac{k\lambda}{\beta \cos\theta}$$

where: D is the mean crystalline size of the particle, *K* is a dimensionless shape factor. The shape factor has a typical value of about 0.9 but varies with the actual shape of the crystallite. λ is the wavelength(1.5418 A^0) of X- ray radiation source. β is the line broadening at half the maximum intensity (FWHM), after subtracting the instrumental line broadening, in radians and θ is the Bragg angle[35,37].

iii) FTIR Analysis: FTIR spectra(Fig.1:c & d) of synthesized AgNps and methanolic crude *R emodi* root extract show transmittance at 3387 cm^{-1}, 2921 cm^{-1}, 1618 cm^{-1}, 1449 cm^{-1}, 1267 cm^{-1}, 10.27 cm^{-1}, 630 cm^{-1} and 3432 cm^{-1}, 2924.8 cm^{-1}, 1625 cm^{-1}, 1384 cm^{-1}, 1056 cm^{-1}, 621 cm^{-1} respectively. Variation in trasmission intensities of both synthesized AgNps and crude root extract were observed which is because of interaction of nanoparticles with biomolecules [35, 37].

FTIR spectrum of AgNPs synthesized uisng *R. emodi* shows the absorbance peaks at 3432 cm^{-1} indicating polyphenolic group along with 621 cm^{-1} aromatic C-H and the sharp peak at 2924 cm^{-1} may be due to C-H stretching of methylene group. Further, peaks at 1028 cm^{-1} - 1236 cm^{-1} indicate C-O single bond, peak at 1625 cm^{-1} represent carbonyl group (-C=O) from polyphenols. The band at 1384 cm^{-1} in AgNPs may be attributed to -C-O stretching mode. The band arising at 818 cm^{-1} is due to bending vibration of C-O-S. The absorption band at 1511 cm^{-1} corresponds to C=O stretching (carboxylic or amide) which is shifted (from 1511 cm^{-1} to 1625 cm^{-1}) after reaction. A strong peak at 3387 cm^{-1} represents primary N-H stretching. The bands at 1448 cm^{-1}, 1161 cm^{-1}, 1071 cm^{-1} are associated with respect to the non-conjugated C=C stretching, C-C stretching, and vibrations of the chain -C-O-C. The absorption peak at around 757 cm^{-1} indicates the aromatic banding. The majority of FTIR bands showed the characteristics functional groups of phenol, alcohol, aldehydes which reflects the presence of anthraquinones in *R emodi* roots. These anthraquinones may be involved in capping and stabilizing the AgNps.

iv) TEM with EDX: The size and morphology of synthesized AgNps were examined by EDX combined with TEM (Fig.2: a, b & e). The size of the AgNps was found to be in the range of 10-40 nm. TEM analysis indicated polydispersed shape of the particles. The EDX spectrum shows signals for silver and copper. The intense signals observed at 3KeV indicate that Ag was the major element of synthesized nanoparticles. It was because of the optical absorption of silver due to the surface plasma resonance in the range. The other signal of carbon indicates the presence of plant extract, which represents to the biomolecules capping over AgNp's. Copper metal peak is due to grid used for analysis. [41]

v) FE-SEM Analysis: The FE-SEM images (Fig.2: c & d) images further indicated that the synthesized AgNps were spherical in shape with mild agglomeration.

3C. Antimicrobial Investigation

Antibacterial and antifungal activity of the methanolic *R emodi* root extract and of the synthesized AgNPs were investigated (Fig.:3) The antimicrobial activity was examined by agar well diffusion assay which indicated well defined zones of inhibition, diameter in mm against three Gram +ve bacterial species namely *Bacillus subtilis, Staphylococcus aureus* and *Streptomyces griseous*; one Gram –ve *E coli;* and two fungal species *Aspergillus niger* and *Fusarium oxysporum.* It was observed that synthesized AgNps exhibit significant inhibitory activity against all bacterial and fungal species as compared to crude root extract. The synthesized AgNps showed prominent results against *Bacillus subtilis* (22±0.5 mm), *E Coli* (30±0.5 mm), *Staphylococcus aureus* (34±0.5 mm) and moderate activity against *Streptomyces griseous*(16±0.5 mm), *Aspergillus niger* (14±0.5 mm), *Fusarium oxysporum*(20±0.5 mm) at 100 mg/ml concentration on comparison to inhibition zone by crude extract(Table-1).

The exact mechanism behind the elevated antimicrobial activity is still not clearly known and debatable. It can be assumed that the surface area to volume ratio of nanoparticles is playing a crucial role in furnishing antimicrobial activity against microbes. The effect of capping is due to the presence of phytochemicals around nanoparticles gives particular type of surface functionality to behave in a specific way to different cell types [37]. C. Raj Kuberan et al. [35] reported three possible mechanisms of AgNps against microbes, (i) it is believed that Ag^+ interferes with bacterial cell membrane synthesis, (ii) AgNps interfere with thio group of bacterial cell affecting respiratory chain reaction, cell division and finally leads to death, (iii) AgNps release silver ions that ill penetrate to the cell wall causing condensation of DNA damage and also by affecting the protein synthesis. Morones et al. [38] proposed that silver act as soft acid which acts upon the sulphur and phosphorous bases of DNA and inactivates its replication thus disabling the nuclear machinery of the cell. I. Sonadi et al. [39] mentioned that silver nanoparticles has ability to attach with the bacterial membrane causing structural changes in its membrane leading to the formation of 'pits' where they accumulate.

Hence, the involvement of above listed mechanisms might be responsible for elevated antimicrobial activity of AgNps.

3D. Cytotoxicity of silver nanoparticles

Crude root extract and synthesized AgNps were taken for cytotoxicity (Table-2) against MCF-7 cell lines using MTT [3-(4, 5- dimethyl thiazol-2-yl)-2, 5 diphenyl tetrazolium bromide] assay. It was observed that synthesized AgNps induce cytotoxicity against MCF-7 cell lines in dose dependent manner [50, 100, 150, 200 and 250µg/ml]. Significant cytotoxicity was reported in biosynthesized AgNps than crude extract with LC_{50} 250µg/ml with respect to positive control. Morphological changes in cells treated with different

concentration of AgNps were observed due to apoptosis. The recorded and captured images of the same are mentioned in Fig.:4.

In earlier study AgNps synthesized using *Calotropis gigantean* latex has shown significant anticancerous effect against HeLa cell lines [35]. It was proved [40] that AgNps have antiangiogenic properties and exert ability to block the activity of abnormally expressing signal protein. D. Nayak [37] reported that biosynthesized silver nanoparticles synthesized using different plant origin *Cucurbita maxima* (petals), *Moringa oleifera* (leaves), *Acorus calamus*(rhizome) show significant antimicrobial and anticancer potential. It is because of nano size regime, the silver nanoparticles may directly bind to the DNA of the pathogenic bacterial strains leading to higher antimicrobial and anticancer potential. S. Arokiyaraj et al. [41] reported rapid green synthesis of silver nanoparticles from *Chrysanthemum indicum* L and its antibacterial and cytoxic effects. They have used MTT and LDH assays to observe cytotoxic potential of AgNps.

J.R. Nakkala et al. [42] mentioned cytotoxic effects shown by AgNps in HeLa and A549 cells involved apoptotic changes. These findings emphasize that synthesized nanoparticles may find their application in the field of nanomedicine.

4. CONCLUSION

AgNPs were synthesized using root crude extract of *R. emodi*. UV-Visible absorbance spectral analysis confirmed the surface Plasmon resonance of AgNPs. FTIR analysis reflects the presence of anthraquinones which are responsible for capping and stability of nanoparticles. XRD, FE-SEM, TEM with EDX analysis confirmed the crystalline nature of the synthesized biogenic AgNPs with spherical shape, size in the range 10-40 nm (average size 27.5 nm). Biogenic AgNPs had shown significant antibacterial and antifungal potential. Cytotoxic potential of synthesized AgNps was found better against breast cancer cell lines (MCF-7). The results obtained in this study advocate the use of biosynthesized nanoparticles in Nano medicine applications with more understanding of its mode of action.

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Research Article

AUGMENTED ECCENTRIC CONNECTIVITY INDEX OF POLYCYCLIC AROMATIC HYDROCARBONS (PAH_K)

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ABSTRACT

Let G be a molecular graph in which the set of vertices represents the atoms and the set of edges corresponds to the bonds. The *Eccentric Connectivity index ξ(G)* is equal to $\xi(G) = \sum_{u \in V(G)} d_u \times \varepsilon(u)$, where $\varepsilon(u)$ referred as the length of a maximal path connecting a vertex *u* to another vertex of *G* and d_u referred as the degree of the vertex *u*. The Augmented eccentric connectivity index of a connected graph *G* is defined as ${}^{A}\xi(G) = \sum_{v \in V(G)} \frac{M(u)}{\varepsilon(u)}$, where *M (u)* denotes the product of degrees of all neighborhood of vertex *u.* In this paper, the augmented eccentric connectivity index for Polycyclic Aromatic hydrocarbons (PAH_k) has been computed.

Keywords: Molecular graph, Eccentric connectivity index, augmented eccentric connectivity index, Polycyclic Aromatic hydrocarbons (PAH_k).

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1. INTRODUCTION

Let G be a molecular graph in which the vertex and the edge sets are represented by V(G) and E(G), respectively, the vertices of the graph represents the atoms of molecules and the edges corresponds to the chemical bond. Let d_u denote the degree of a vertex in G. If $d_u = 1$ then u is said to be a pendent vertex. For two vertices u,v, $d(u,v)$ defines the length of the shortest path connecting u and v. The eccentricity of a vertex u is defined to be

$$ecc(u) = \max\ \{ d(v,u) :\ v \in V(G)\}.$$

A topological index TI is a number that is invariant under the Aut(G). A variety of TIs have been proposed for characterization of chemical structures and used for structure property correlations in QSPR models [1-3]. The eccentric connectivity index of a graph G was proposed by Sharma, Goswami and Madan [4]. A generalization of eccentric connectivity index, known as augmented eccentric connectivity index of a graph G was proposed by Dureja and Madan [5], also the eccentric connectivity index for an infinite family of linear Polycene parallelogram Benzenoid P(n,n) ($\forall$ n≥1) was computed in [6] while MEC index for the same structure was computed in [7] and the Ediz eccentric connectivity index of P (n, n) was computed in [9].

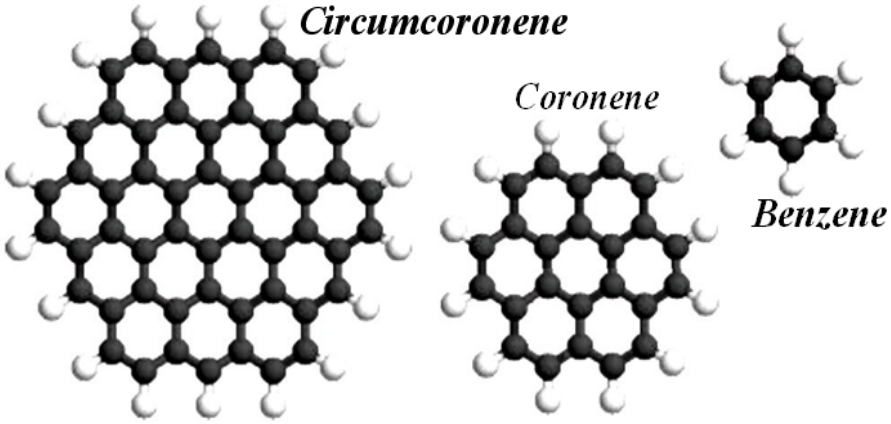
Polycyclic Aromatic hydrocarbons considered here is a family of hydrocarbons which contains several copies of benzene C_6 and play an important role in graphitization of organic materials [10]. For structural detail and tuning of molecular properties toward specific application one can consult [11-17]. See some properties and some useful information about this family readers are encourage to see [18-27].

The first famous members of this hydrocarbon family (PAH family) are denoted and shown as follow

PAHk is structured as:

- For *k=1* we have *Benzene* with six carbon (C) and six hydrogen (H) atoms,
- For *k=2*, *Coronene* with 24 carbon and twelve hydrogen atoms,
- For *k=3 Circumcoronene* with 54 carbon and eighteen hydrogen atoms.

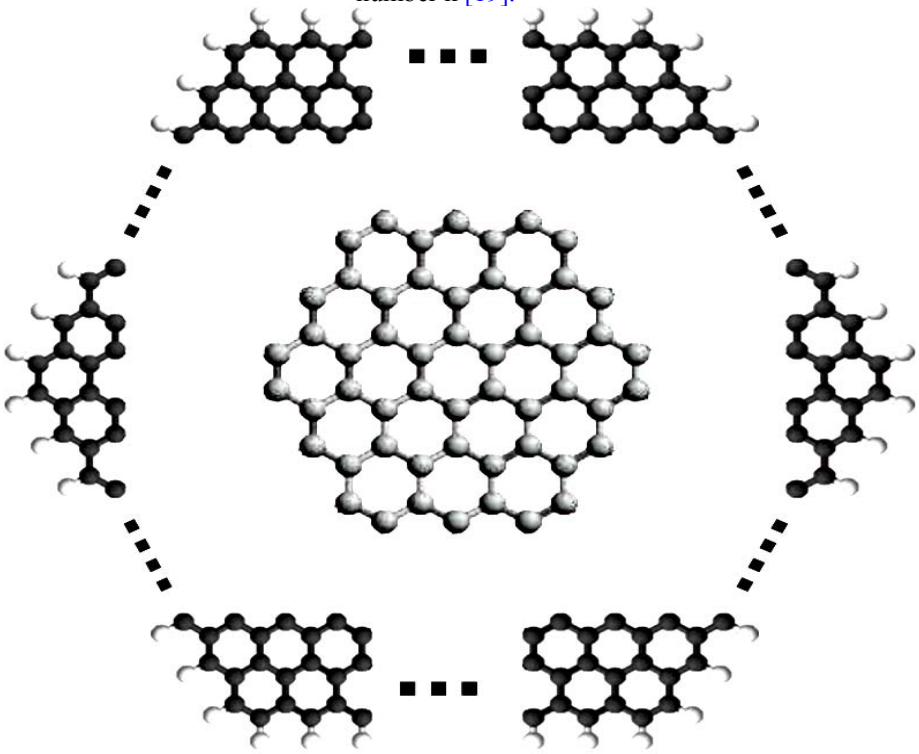
Figure 1: The some first members Benzene, Coronene, Circumcoronene of the Polycyclic Aromatic Hydrocarbons PAH_k family [18-27].



2. RESULTS AND DISCUSSION

In this section, we computed the augmented eccentric connectivity index of polycyclic aromatic hydrocarbons (PAH_k). The PAH_k can be thought as small pieces of graphene sheets with the free valences of the dangling bonds saturated by *H*. A graphene sheet can be interpreted as an infinite PAH molecule. Successful utilization of PAH molecules in modeling graphite surfaces has been reported earlier. Figure 2 represent the general representation of polycyclic aromatic hydrocarbons (PAH_k).

Figure 2: The general representation of polycyclic aromatic hydrocarbon PAH_k for all integer number k [19].



Theorem 1: The Augmented Eccentric Connectivity index of PAH_k is

$$^{A}\xi(G) = \frac{1980k^2 + 261k - 27}{(4k-1)(8k+2)} + \sum_{i=1}^{k-1} \frac{162i\,(4k+4i-1)}{4i^2 + (4i+1)2k - 2i}$$

***Proof**:* Consider the general representation of PAHs having $6n^2$ carbons and $6n$ hydrogen atoms We use *Ring cut method* for Circumcoronene homologous series of Benzenoid and denote all vertices of degree three of PAH_n by β and γ and all vertices of degree one by α are:

$$V(PAH_n) = \{\alpha_{z,l}\ ,\ \beta^i_{z,l}\ ,\ \ \gamma^i_{z,j}\ : l = 1,...,k\ ,\ \ j \in Z_i\ ,\ l \in Z_{i-1}\ \&\ z \in Z_6\}$$

See Figure 3, where $Z_i = \{1, 2, ..., i\}$ is the cyclic finite group of order *i*.

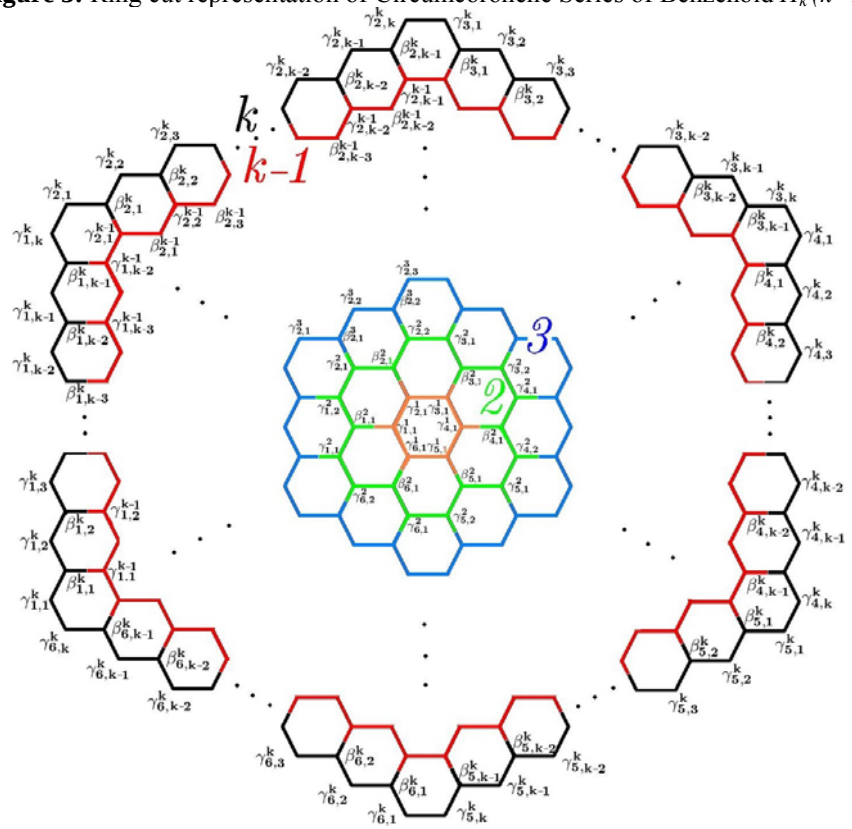
We divide the all the vertices such that i^{th} ring-cut consist of vertices $\beta^i_{z,j}$ $\gamma^i_{z,j}$ $(\forall i = 1, ..., k; z \in Z_6$, $j \in Z_i)$ and size of the ring cut is $6i + 6(i-1)$, the common property of a ring cut is their farthest vertices also $d(\gamma^i_{z,j}, \gamma^k_{z,j}) = d(\beta^i_{z,j}) = 2(k-i)$. By ring cuts of PAH_n we have the following eccentricities [27-30] of the corresponding vertices i.e

i. $$\varepsilon(\beta^i_{z,j}) = d(\beta^i_{z,j}, \beta^i_{z+3,j}) + d(\beta^i_{z+3,j}, \gamma^k_{z+3,j}) + d(\gamma^k_{z+3,j}, \alpha_{z+3,j}) = 2k + 2i - 1$$
For all vertices $\beta^i_{z,j}$ of PAH_k $(\forall i = 1, ..., k;\ z \in Z_6;\ \ j \in Z_{i-1})$

ii. $$\varepsilon(\gamma^i_{z,j}) = d(\gamma^i_{z,j}, \gamma^i_{z+3,j}) + d(\gamma^i_{z+3,j}, \gamma^k_{z+3,j}) + d(\gamma^k_{z+3,j}, \alpha_{z+3,j}) = 2(k+i)$$
For all vertices $\gamma^i_{z,j}$ of PAH_k $(\forall i = 1, ..., k;\ z \in Z_6;\ \ j \in Z_{i-1})$

iii. $$\varepsilon(\alpha_{z,j}) = d(\gamma^i_{z,j}, \gamma^i_{z+3,j}) + d(\gamma^i_{z+3,j}, \gamma^k_{z+3,j}) + d(\gamma^k_{z+3,j}, \alpha_{z+3,j}) = 4k + 1$$
For all vertices $\alpha_{z,j}$ (hydrogen atoms) of PAH_k $(\forall j = 1, ..., k;\ z = 1, ..., 6$

Figure 3: Ring cut representation of Circumcoronene Series of Benzenoid H_k *(k>1).*



and compute *M (u)* for every vertex *u* of PAH_n as under:

1. $\forall j \in Z_k, \forall z \in Z_6;\ M(\gamma^k_{z,j}) = 3\times 3\times 1 = 9$; since $\forall \gamma^k_{z,j}; d_{\beta^k_{z,j-1}} = d_{\beta^k_{z,j}} = 3$ and $d_{\alpha_{z,j}} = 1$
2. $\forall j \in Z_{k-1}, \forall z \in Z_6;\ M(\beta^k_{z,j}) = 3\times 3\times 3 = 27$; since $\forall \beta^k_{z,j}; d_{\gamma^k_{z,j+1}} = d_{\gamma^k_{z,j}} = 3$ and $d_{\beta^{k-1}_{z,j}} = 3$
3. $\forall j \in Z_i, , i \in Z_k, \forall z \in Z_6;\ M(\beta^i_{z,j}) = M(\gamma^i_{z,j}) = 3\times 3\times 3 = 27$; and $d_u = 3$ since $\forall u \in V(H_k) - \{\gamma^k_{z,j} : j \in Z_i, \forall z \in Z_6\}$
4. $\forall j \in Z_k, \forall z \in Z_6;\ M(\alpha_{z,j}) = 3$; since $\forall \gamma^k_{z,j}\ \ d_{\gamma^k_{z,j}} = 3$

It is obvious that all vertices in (1&2) are considered from the i^{th} ring-cut and vertices in (3) are from ring-cuts $1,...,i-1$. Therefore the AECI of PAH_n is

$$
\begin{aligned}
{}^A\xi(PAH_n) &= \sum_{u\in V(PAH_n)} \frac{M(u)}{\varepsilon(u)}, \\
&= \sum_{\gamma^k_{z,j}} \frac{M(\gamma^k_{z,j})}{\varepsilon(\gamma^k_{z,j})} + \sum_{\beta^i_{z,j}} \frac{M(\beta^i_{z,j})}{\varepsilon(\beta^i_{z,j})} + \sum_{\gamma^{ik}_{z,j}\in(PAH_{k-1})} \frac{M(\gamma^i_{z,j})}{\varepsilon(\gamma^i_{z,j})} + \sum_{\alpha_{z,j}} \frac{M(\alpha_{z,j})}{\varepsilon(\alpha_{z,j})} \\
&= \sum_{\gamma^k_{z,j}} \frac{9}{4k} + \sum_{i=1}^{k}\sum_{j=1}^{i}\sum_{z=1}^{6} \frac{27}{2k+2i-1} + \sum_{i=1}^{k-1}\sum_{j=1}^{i}\sum_{z=1}^{6} \frac{27}{2(k+i)} + \sum_{\alpha_{z,j}} \frac{3}{4k+1} \\
&= 6k.\frac{9}{4k} + 6k.\frac{3}{4k+1} + [\sum_{i=1}^{k-1} \frac{162i(4k+4i-1)}{4i^2+(8i+2)k-2i} + \frac{162k}{4k-1}] \\
&= \frac{171k+27}{8k+2} + \frac{162k}{4k-1} + [\sum_{i=1}^{k-1} \frac{162i(4k+4i-1)}{4i^2+(8i+2)k-2i} + \frac{162k}{4k-1}] \\
&= \frac{1980k^2+261k-27}{(4k-1)(8k+2)} + \sum_{i=1}^{k-1} \frac{162i(4k+4i-1)}{4i^2+(8i+2)k-2i}
\end{aligned}
$$

which completes the proof. ■

Example 1: The Augmented eccentric connectivity index ${}^A\xi(PAH_2)$ is

$$
\begin{aligned}
{}^A\xi(PAH_2) &= \sum_{u\in V(PAH_2)} \frac{M(u)}{\varepsilon(u)}, \\
&= \sum_{\gamma^k_{z,j}} \frac{M(\gamma^2_{z,j})}{\varepsilon(\gamma^2_{z,j})} + \sum_{\beta^i_{z,j}} \frac{M(\beta^2_{z,1})}{\varepsilon(\beta^2_{z,1})} + \sum_{\gamma^{ik}_{z,j}\in(PAH_{k-1})} \frac{M(\gamma^2_{z,j})}{\varepsilon(\gamma^2_{z,j})} + \sum_{\alpha_{z,j}} \frac{M(\alpha_{z,j})}{\varepsilon(\alpha_{z,j})} \\
&= 12\ (\frac{9}{8}) + 6(\frac{27}{5}) + 6(\frac{27}{6}) + 12(\frac{3}{9})\ = 76.9\ .
\end{aligned}
$$

4. CONCLUSION

In this paper an augmented eccentric connectivity index for polycyclic Aromatic hydrocarbons is computed. These types of graphs are the generalization of C_6 which is frequently used in chemistry physics and Nanoscience and is very useful to synthesize the aromatic compounds.

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