EUROPEAN Journal of Molecular Biotechnology

Has been issued since 2013.
ISSN 2310-6255. E-ISSN 2409-1332
2017. 5(2). Issued 2 times a year

Journal is indexed by: **Chemical Abstracts Service** (USA), **CiteFactor** – Directory of International Reseach Journals (Canada), **Cross Ref** (UK), **EBSCOhost Electronic Journals Service** (USA), **Global Impact Factor** (Australia), **International Society of Universal Research in Sciences** (Pakistan), **Journal Index** (USA), **Electronic scientific library** (Russian Federation), O**pen Academic Journals Index** (Russian Federation), **Sherpa Romeo** (Spain), **ULRICH's WEB** (USA), **Universal Impact Factor** (Australia).

All manuscripts are peer reviewed by experts in the respective field. Authors of the manuscripts bear responsibility for their content, credibility and reliability.

Editorial board doesn't expect the manuscripts' authors to always agree with its opinion.

Postal Address: 1367/4, Stara Vajnorska str., Bratislava – Nove Mesto, Slovakia, 831 04

Website: http://ejournal8.com/
E-mail: aphr2010@mail.ru

Founder and Editor: Academic Publishing House Researcher s.r.o.

Release date 15.12.17.
Format 21 × 29,7/4.

Headset Georgia.

Order № 16.

European Journal of Molecular Biotechnology

2017

Is. 2

CONTENTS

Articles and Statements

Published in the Slovak Republic
European Journal of Molecular Biotechnology
Has been issued since 2013.
ISSN: 2310-6255
E-ISSN: 2409-1332
2017, 5(2): 52-59

DOI: 10.13187/ejmb.2017.2.52
www.ejournal8.com

Articles and Statements

Synthesis, spectroscopic NMR and theoretical (HF and DFT) investigation of 3,5,5,9-tetramethyl-2-nitro-6,7,8,9-tetrahydro-5H-benzocycloheptene and 2,5,9,9-tetramethyl-1,3-dinitro-6,7,8,9-tetrahydro-5H-benzocycloheptene

N. Ourhriss [a, b], A. Zeroual [b, c, *], C. A. Gadhi [d], A. Benharref [b], A. Abourriche [a], A. Bennamara [a], A. ElHajbi [c]

[a] Biomolecular and Organic Synthesis Laboratory, Faculty of Sciences Ben M'sik Casablanca, Hassan II University, Morocco
[b] Laboratory of Biomolecular Chemistry, Natural Substances and Reactivity, URAC 16, Faculty of Sciences Semlalia, Cadi Ayyad University, P.O. Box 2390, Marrakech, Morocco
[c] Laboratory of Physical Chemistry, Faculty of Science, Chouaïb Doukkali University, P.O. Box 20, 24000 El Jadida, Morocco
[d] Laboratory of Biotechnology, Valorization and Protection of Vegetable Resources, Faculty of Sciences Semlalia, Cadi Ayyad University, P.O. Box 2390, Marrakech, Morocco

Abstract

3,5,5,9-tetramethyl-2-nitro-6,7,8,9-tetrahydro-5H-benzocycloheptene and 2,5,9,9-tetramethyl-1,3-dinitro-6,7,8,9-tetrahydro-5H-benzocycloheptene has been synthesized using nitration reaction of ar-himachalene. Also, optimized geometry of the title compound are evaluated using HF and DFT/B3LYP with 6-31(d) basis set 6-31G(d) methods. Moreover, the 13C NMR chemical shift values of the molecule (2,5,9,9-tetramethyl-1,3-dinitro-6,7,8,9-tetrahydro-5H-benzocycloheptene) are calculated using HF and DFT/B3LYP/6-31G(d) and compared with experimental results. The global of the reactivity indices and Parr functions of the reagents were calculated by HF and DFT to identify the actives regions of these molecules, we found that these reactions are regiospecific.

Keywords: Nitration reaction, ar-himachalene, density functional theory (DFT) and Hartree Fock (HF).

1. Introduction

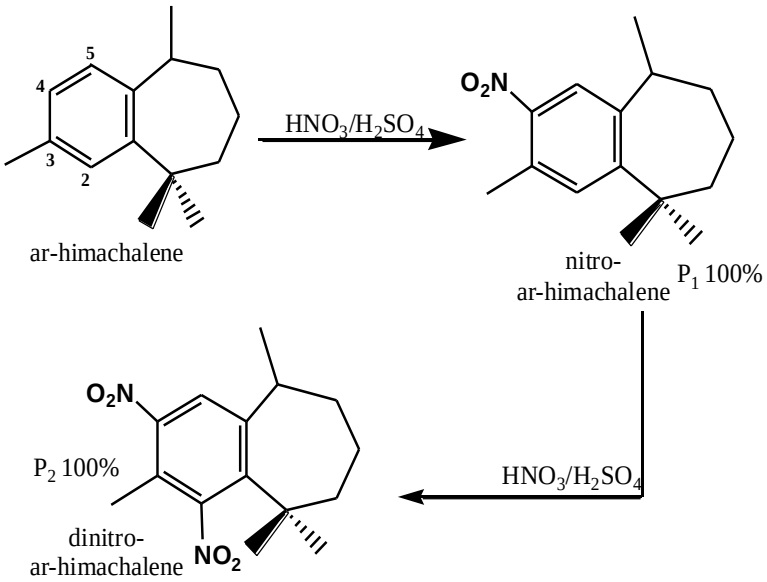
The bicyclic sesquiterpenes α- and β-himachalene are the main constituents of the essential oil of Atlas cedar (Cedrus atlantica) (Joseph et al, 1968; Plattier et al, 1974). The reactivity of these sesquiterpenes and their derivatives has been studied extensively by our team in order to prepare new products with biological properties (Lassaba et al, 1998; Chekroun et al, 2000; El Jamili et al, 2002; Dakir et al, 2004). Indeed, these compounds have been tested, using the food poisoning technique, for their potential antifungal activity against the phytopathogen Botrytis cinerea (Daoubi et al, 2004). Thus, the catalytic dehydrogenation of the mixture of α- and β-himachalene

*Corresponding author
E-mail addresses: zeroual19@yahoo.fr (A. Zeroual)

by 5% of palladium in the charbone (10%), at 150 ° C., we obtained aryl-himachalene with a good yield, (Daunis et al, 1981). Treatment of aryl-himachalene with 1 equivalent and then with 2 equivalents of nitric acid in the presence of sulfuric acid, leads for 4 hours at room temperature, to compounds 1 and 2 (Scheme 1).

In latest years, computational chemistry has become a principal instrument for chemists and a well-accepted instrument for experimental chemistry.

Herein, in order to understand the molecular mechanism and the regioselectivity of the nitration reaction ar-himachalene (scheme 1), a theoretical characterization of the molecular mechanism of this nitration reaction is carried out within the Computational methods. We examine analyses of the reactivity indices of the reagents, Parr functions of ar-himachalene using Hartree Fock (HF) and density functional theory (DFT) methods with 6-31G(d) basis set. Our aim is to explicate the regioselectivity experimentally obtained.



Scheme 1 the nitration reaction of ar-himachalene, 2-nitroar-himachalene.

2. Experimental

General procedure for the preparation of products

In a reactor of 250 ml volume equipped with a magnetic stirrer and a dropping funnel, were introduced 60 ml of dichloromethane, 3 ml of nitric acid and 5 ml of concentrated sulfuric acid. After cooling, (30 mmol) of ar-himachalene, dissolved in 30 ml of dichloromethane was added dropwise through the dropping funnel. The reaction mixture was stirred for 4 h, then quenched with 50 ml of water ice and extracted with dichloromethane. The organic layers were combined, washed five times with 40 ml with water and dried over sodium sulfate and then concentrated under vacuum. Chromatography on a silica gel column of the residue with hexane–ethyl acetate (96/4) as eluent of the residue gave the title compounds (66%; 20 mmol).

3,5,5,9-Tetramethyl-2-nitro-6,7,8,9-tetrahydro-5H-benzocycloheptene, yellow oil, 1H NMR δ: 1,2 (3H, m,CH3), 1.24 (2H, m, CH2), 1.25- 1,35 (6H, s, CH3), 1.46

(3H, d, J 6.9, CHCH3), 1.70 - 1.85 (4H, m, CHH), 2.45 (3H, s, CH3), 3,2 (1H, m, CHCH3), 2,48 (2H, s,ArCH2), 7.71 (1H, s, ArH), 7,75 (1H, s, ArH); 13C NMR δ: 20.79, 21.09, 24.18, 30, 34.14, 34.78, 36.42, 40.29, 40.97,122.21, 130.93, 131.48, 143.86, 147.25, 154.03. EIMS, m/z 247.1 (M+,100),232.1(68.5),230.1 (28), 144.1 (18), 41.1 (19). HRMS, 247.0288.

2,5,9,9-Tetramethyl-1,3-dinitro-6,7,8,9-tetrahydro-5H-benzocycloheptene, yellow oil, 1H NMR δ: 1,2 (3H, m, CH3), 1.24 (2H, m, CH2), 1.25 - 1,35 (6H, s, CH3), 1.46 (3H, d, J 6.9, CHCH3), 1.70 - 1.85 (4H, m, CHH), 2.45 (3H, s, CH3), 3,2 (1H, m, CHCH3), 2,48 (2H, s,ArCH2), 7,8 (1H, s, ArH); 13C NMR δ: 14.34, 20.4, 22.9, 30.34, 31.63, 34.48, 35.49, 42.51, 44.61, 122.43, 123.62, 132, 143.22, 146.38, 149. EIMS, m/z 292.1 (M+, 100), 248.1 (86), 232.1 (61.5), 230.1 (32), 144 (21), 41.1 (19). HRMS, 292.1090.

3. Computational methods

The equilibrium geometries have been optimized at the B3LYP/6-31G (d) calculation level on Gaussian 09 (Frisch et al, 2009), using Berny's algorithm (Schlegel, 1982). Atomic electronic populations and reactivity indices were calculated using natural population (NPA). The global electrophilicity index (Parr et al, 1999) ω, was given by the following expression $\omega = \frac{\mu^2}{2\eta}$, in terms of the electronic chemical potential μ and the chemical hardness η. Both quantities could be approached in terms of the one-electron energies of the frontier molecular orbital HOMO and LUMO, ε_H and ε_L as $\mu = \frac{\varepsilon_H + \varepsilon_L}{2}$ and $\eta = \varepsilon_H - \varepsilon_L$, respectively. The empirical nucleophilicity index N (Domingo et al, 2009) based on the HOMO energies obtained within the Kohn-Sham (Kohn *et al.*, 1965), and defined as $N = E_{HOMO}(Nu) - E_{HOMO}(TCE)$. the nucleophilicity was referred to tetracyanoethylene (TCE). Electrophylic P_k^+ and nucleophilic P_k^- Par functions were obtained through analysis of the Mulliken atomic spin density (ASD) of the radical anion and radical cation of the reagents. (El Haib et al, 2018; Zeroual et al, 2017; Zoubir et al, 2017; El Idrissi et al, 2017; Ourhriss et al, 2017; El Idrissi et al, 2017; Zeroual et al, 2015; Barhoumi et al, 2015; Ryachi et al, 2015).

4. Discussion

This section was divided into three parts: (1) experimental result, (2) next, an analysis of the reactivity indices of the reagents. (3) After that, predicting the regioselectivite for carrying out the aromatic nitration reaction of ar-himachalene, (3) finally, the 1H and 13C NMR chemical shift values of the molecule are calculated and compared with experimental results.

4.1. Comparative analysis of the conceptual DFT indices of the reagents

The global HF and DFT indices, namely, HOMO, LUMO, chemical hadness η, the electronic chemical potential μelectrophilicity w and nucleophilicity N, are given in table 1.

Table 1. HF and DFT at B3LYP/6-31G(d) HOMO, LUMO, chemical hardness, electronic chemical potential, electrophilicity and nucleophilicity in eV, of the ar-himachalene, 2-nitroar-himachalene (3,5,5,9-Tetramethyl-2-nitro-6,7,8,9-tetrahydro-5H-benzocycloheptene) and nitric acid.

	method	HOMO	LUMO	η	μ	w	N
Ar-himachalene	**DFT**	-5.953	0.153	6.106	-2.899	0.688	3.578
	HF	-8.138	3.923	12.061	-2.107	0.184	4.369
2-nitroar-himachalene	**DFT**	-6.702	-2.093	4.608	-4.397	2.098	2.829
	HF	-8.974	1.617	10.592	-3.678	0.638	3.533
Nitric acid	**DFT**	-8,179	-2,179	5,999	-5,179	2,235	1,352
	HF	-13,32	2,793	16,114	-5,263	0,859	-0,812

HOMO (TCE) (DFT=-9.532 and HF=-12.508)

Given the values offered in Table 1, the higher electronic chemical potential of ar-himachalene and 2-nitroar-himachalene find by DFT and HF methods, -2.899, -4.397 and -2.107, -3.678 eV respectively, than that of nitric acid, -5,179 (DFT), -5,263 (HF) eV, indicates along the interaction between these compounds the GEDT, as a determine of reaction polarity, have to take place from former toward latter which, respectively, act as nucleophile and electrophile ar-himachalene and 2-nitroar-himachalene has a low global electrophilicity w index, (0.688, 2.098) and (0.184, 0.638eV), and a elevated nucleophilicity N index, (3.578, 2.829) and (4.369, 3.533eV), being classified as weak electrophile and a strong nucleophile within the electrophilicity and nucleophilicity scales, respectively. On the other hand, nitric acid exhibits an elevated global electrophilicity w index, 2,235, 0,859eV, and a weak nucleophilicity N index, 1,352, -0,812eV, being classified as a strong electrophile but a low nucleophile.

4.2. The nucleophilic P_k^- Parr functions of the ar-himachalene and 2-nitroar-himachalene.

As the non-symmetric reagents, the initial two-center interaction between the most electrophilic center of the electrophile and the most nucleophilic center of the nucleophile. The nucleophilic P_k^- Parr functions, as powerful tools in the study of the local reactivity in polar processes. Therefore, the nucleophilic Parr functions of the ar-himachalene, and 2-nitroar-himachalene were examined in order to differentiate the most electrophilic and nucleophilic centers of the species involved in these nitration reactions and, thus, to explicate the regioselectivity experimentally observed (Fig. 1).

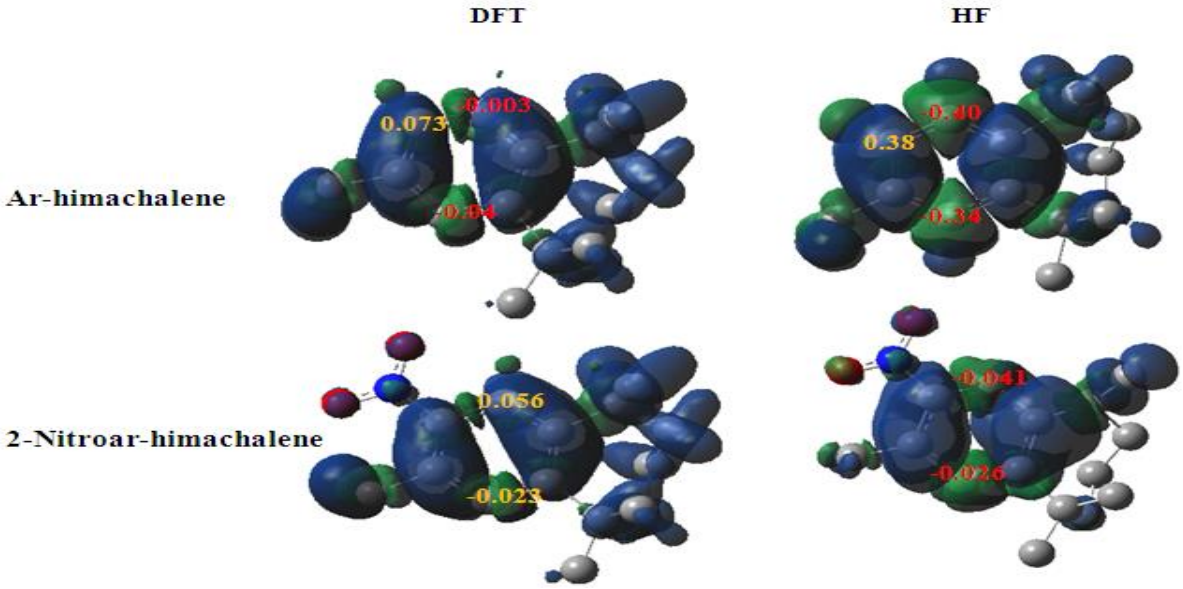


Fig. 1. 3D representation of the nucleophilic P_k^- Parr functions maps of the ar-himachalene and 2-nitroar-himachalene obtained by HF and DFT.

We can observed from figure 1 that the C_4 carbon of the ar-himachalene molecule has a greater nucleophilic P_k^- Parr functions value of $P_4^- = 0.073$ obtained by DFT calculation than the other atoms $P_2^- = -0.04$ and $P_5^- = -0.003$, on the other hand the nucleophilic P_k^- Parr functions obtained by HF method indicate clearly that ar-himachalene have only one nucleophilic center C_4 carbon, $P_4^- = 0.38$. We conclude that the best interaction will be between the carbon atom C_4 of ar-himachalene and the nitro group in good agreement with experience. We can observe that the nucleophilic P_k^- Parr functions of the C_2 carbon of the 2-nitroar-himachalene obtained by DFT $(P_2^- = -0.023)$ small than the C_5 carbon $(P_5^- = 0.056)$, in opposite the result obtained by HF method indicate $(P_2^- = -0.023)$ high than the C_5 carbon $(P_5^- = -0.041)$, indicating that the best interaction between the carbon C2 of 2-nitroar-himachalene and the nitro group, in total conformity with the experimental observations.

4.2. NMR chemical shift analysis

Nuclear magnetic resonance is a discriminating local investigate that can give perfect information about near atomic environments. To perform this study, we employed DFT and HF combined with the GIAO method (Wolinski et al., 1990; Cheeseman et al., 1996), to calculate the isotropic electronic tensors σ_{iso}. The calculation of the isotropic chemical shifts δ_{iso}, are referred to the tetramethylsilane (TMS) and obtained by the relation:

$$\delta_{iso} = -[\sigma_{iso} - \sigma_{ref}]$$

In the present analyze, the theoretical ^{13}C NMR chemical shift values of title compound 2,5,9,9-tetramethyl-1,3-dinitro-6,7,8,9-tetrahydro-5H-benzocycloheptene were calculated by HF and DFT/B3LYP methods with 6-31G(d) basis set using GIAO method in the gas phase (Atoms

labeling is according to Figure 2). Then calculated ^{13}C NMR chemical shifts were compared to the experimental values (Tables 3) and the correlation graphics of theoretical chemical shift values of ^{13}C NMR are illustrated in figure 3.

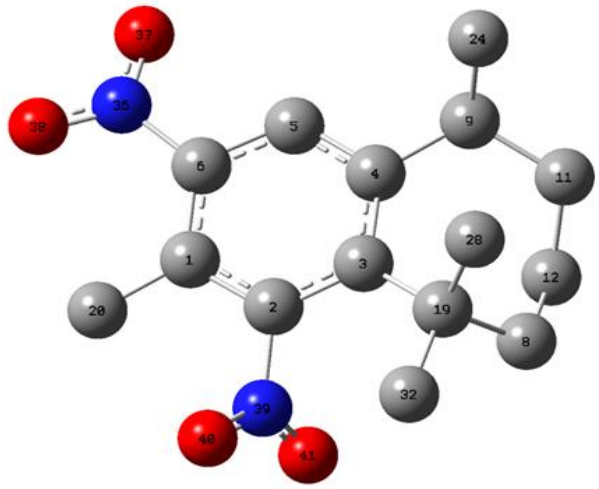


Fig. 2. The theoretical geometric structures of the 2,5,9,9-tetramethyl-1,3-dinitro-6,7,8,9-tetrahydro-5H-benzocycloheptene

Table 3. Theoretical and experimental ^{13}C isotropic chemical shifts for the title compound 2,5,9,9-tetramethyl-1,3-dinitro-6,7,8,9-tetrahydro-5H-benzocycloheptene (Atoms labeling is according to Figure 2).

Carbon number	HF	DFT	Experimental chemical shifts
C20	21.85	13.88	14.34
C12	22.62	30.12	20.40
C24	24.84	29.01	22.9
C32	28.38	34.08	30.34
C11	28.65	36.07	31.63
C28	32.11	38.57	34.48
C19	32.63	44.50	35.49
C8	35.08	43.61	42.51
C9	37.57	48.34	44.61
C1	134.08	126.78	122.43
C5	139.54	131.35	123.62
C4	139.91	143.00	132.00
C6	142.62	142.94	143.22
C2	152.10	154.01	146.38
C3	153.31	145.06	149.00

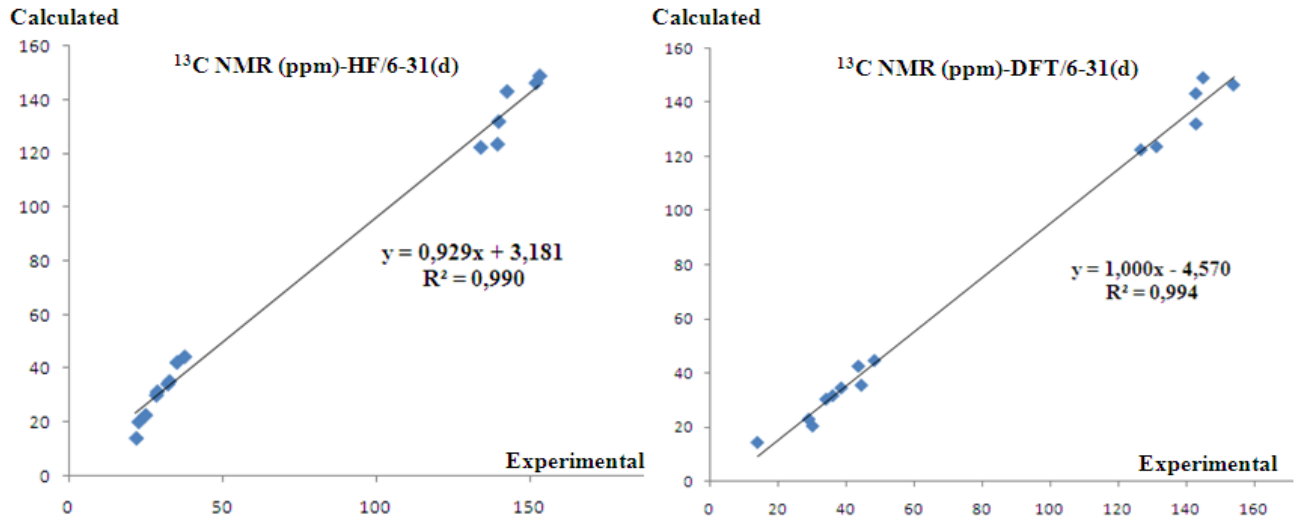


Fig. 3. Correlation graphics of theoretical chemical shift values of ^{13}C NMR of the title compound 2,5,9,9-tetramethyl-1,3-dinitro-6,7,8,9-tetrahydro-5H-benzocycloheptene

^{13}C NMR chemical shifts are reported in ppm relative to TMS. According to the results, there can be seen a good agreement between experimental and calculated values. In addition we examined the relation between experimental and theoretical chemical shift values by comparing the experimental and computed results and obtained linear function formula for Figures 2. According to the outcome, the experimental values are in good conformity with the theoretical values by B3LYP/6-31(d) level as compared to HF/6-31(d) level.

5. Conclusion

In conclusion, 3,5,5,9-tetramethyl-2-nitro-6,7,8,9-tetrahydro-5H-benzocycloheptene and 2,5,9,9-tetramethyl-1,3-dinitro-6,7,8,9-tetrahydro-5H-benzocycloheptene has been synthesized using nitration reaction of ar-himachalene, which are extensive importance as potential strong biologically active compounds or pharmaceuticals. In the present study also, the global of the reactivity indices and Parr functions of the reagents have been examined using the DFT/B3LYP and HF methods with 6-31(d) basis set, to understand the high regioselectivity observed in experience. In addition, from the theoretical and experimental ^{13}C NMR chemical shift values, it can be observed experimental values are in good agreement with the theoretical values by DFT/B3LYP/6-31(d) level compared with HF/6-31(d) level.

References

Cheeseman et al., 1996 – *Cheeseman J. R., Trucks G. W., Keith T. A., Frisch M. J.* (1996). A Comparison of Models for Calculating Nuclear Magnetic Resonance Shielding Tensors, *J. Chem. Phys*, 104. 5497-5509.

Chekroun et al., 2000 – *Chekroun, A., Jarid, A., Benharref, A., Boutalib, A.* (2000). Regio- and Stereoselectivity of β-Himachalene Epoxidation by m-CPBA. A Theoretical Study, *Journal of Organic Chemistry*, 65, 4431–4434.

Dakir et al 2004 – *Dakir, M., Auhmani, A., Ait Itto, M. Y., Mazoir, N., Akssira, M., Pierrot, M. Benharref, A.* (2004). Optimisation of Allylic Oxidation of (1S,3R,8R)-2,2-Dichloro-3,7,7,10-tetramethyltricyclo-[6.4.0.01,3]dodec-9-ene, *Synthetic Communications*, 34 (11), 2001-2008.

Domingo et al 2008 – *Domingo L. R., Chamorro E., Pérez P.* (2008). Understanding the Reactivity of Captodative Ethylenes in Polar Cycloaddition Reactions. A Theoretical Study, *J. Org. Chem.* 73, 4615-4624.

Domingo et al 2011 – *Domingo L. R., Pérez P.* (2011). The nucleophilicity N index in organic chemistry, *Org. Biomol. Chem.* 9, 7168-7175.

El Haib et al 2018 – El Haib A., Elajlaoui R., El Idrissi M., Moumou M., Abouricha S., Zeroual A., Benharref A., El Hajbi A. (2018). The mechanism, the chemoselectivity and the regioselectivity of the 1-Benzyl-4-ethynyl-1H-[1,2,3]triazole and 1-Azidomethyl-4-tert-butyl-benzene in [3+2] cycloaddition reactions: a DFT study, *Mor. J. Chem.* 6, (1), 14-21.

El Jamili et al., 2002 – *El Jamili, H., Auhmani, A., Dakir, M., Lassaba, E., Benharref, A., Pierrot M., Chiaroni, A. Riche C.* (2002). Oxydation et addition des dihalocarbènes sur le β-himachalène, *Tetrahedron Letters,* 43, 6645-6648.

El Idrissi et al., 2016 – *El Idrissi, M., El Haib, A., Zoubir, M., Hammal, R., Zeroual, A., EL Hajbi, A.* (2016). Understanding the regioselectivite of the Baeyer-Villiger reaction of bicyclo[4.2.0]octan-7-one and bicyclo[3.2.0]heptan-6-one: A DFT Study, *Journal of Computational Methods in Molecular Design,* 6: 75-79.

El Idrissi et al., 2017 – *El idrissi M., El Ajlaoui R., Zoubir M., Abouricha S., Moumou M., Ourhriss N., Zeroual A., Benharref A., El Hajbi A.* (2017). Theoretical study of the chemo- and regioselectivity toward [3+2] cycloaddition reaction between mesitonitrile oxides and 2-fluoren-9-ylidene-malononitrile, *Journal of Materials and Environmental Sciences*, 8 (10), 3564-3569.

El-Sabbagh et al., 2009 – *El-Sabbagh O. I., Baraka M. M., Ibrahim S. M., Pannecouque C., Andrei G., Snoeck R., Balzarini J., Rashad A. A.* (2009). Synthesis and antiviral activity of new pyrazole and thiazole derivatives, *European Journal of Medicinal Chemistry*, 44 (9), 3746–3753.

Fukui, 1970 – *Fukui K.* (1970). Formulation of the reaction coordinate, *J. Phys. Chem.* 74, 4161–4163.

Joseph, 1968 – Joseph T. C., Dev S. (1968) "Structure of himachalene monohydrochloride and the preparation of trans-himachalenes," Tetrahedron, 24, 3853-3859.

Hassan, 2013 – *Hassan S. Y.* (2013). Synthesis, antibacterial and antifungal activity of some new pyrazoline and pyrazole derivatives. *Molecules*. 18(3), 2683-711.

Kini et al., 2008 – *Kini S., Gandhi A. M.* (2008). Novel 2-Pyrazoline Derivatives as Potential Antibacterial and Antifungal Agents, *Indian J Pharm Sci.* 70(1), 105–108.

Kohn et al., 1965 – *Kohn W., Sham L.* (1965). Self-Consistent Equations Including Exchange and Correlation Effects, *J. Phys. Rev*. 140(4A), 1133-1138.

Lassaba et al., 1998 – *Lassaba, E., Eljamili, H., Chekroun, A., Benharref, A., Chiaroni, A., Riche, C. & Lavergne, J.-P.* (1998). *"Regio and Stereoselective Epoxidation of Cis-himachalenes and Trans-himachalenes," Lavergne Synthetic Communications,* 28, 2641–2651.

Ourhriss et al., 2017 – *Ourhriss, N., Zeroual, A., Ait Elhad, M., Mazoir, N., Abourriche, A., Gadhi, C. A., Benharref, A., El Hajbi*, A. (2017). Synthesis of 1-isopropyl-4,7-dimethyl-3-nitronaphthalene: An experimental and theoretical study of regiospecific nitration, *Journal of Materials and Environmental Sciences,* 8 (4): 1385-1390.

Parr et al., 2009 – *Parr, R. G., Szentpaly, L. V., Liu, S.* (1999). Electrophilicity Index, *J Am ChemSoc,* 121: 1922-1924.

Plattier et al., 1974 – Plattier, M. & Teisseire, P. (1974). Essential oil of the Atlas Cedar, *Recherches*, 19, 131–144.

Schlegel, 1982 – *Schlegel H.B.* (1982). Optimization of Equilibrium Geometries and Transition. Structures, J. *Comput. Chem.*, 2, 214-218.

Tomasi et al., 1994 – *Tomasi J., Persico M.* (1994). Molecular Interactions in Solution: An Overview of Methods Based on Continuous Distributions of the Solvent, *Chem. Rev*. 94, 2027–2094.

Ungureanu et al., 1997 – *Ungureanu M., Mangalagiu I., Grasu G., Petrovanu M.* (1997). Antimicrobial activity of some new pyridazinum compounds. *Annal Pharm Franc*, 55, 69–72.

Wolinski et al., 1990 – Wolinski K., Hilton J. F., Pulay P. (1990). Efficient Implementation of the Gauge-Independent Atomic Orbital Method for NMR Chemical Shift Calculations, *J. Am. Chem. Soc,* 112, 8251-8260.

Zeroual et al., 2015 – *Zeroual, A., Benharref, A., El Hajbi, A.* (2015). Theoretical study of stereoselectivity of the [1+2] cycloaddition reaction between (1S,3R,8S)-2,2-dichloro-3,7,7,10-tetramethyltricyclo[6,4,0,01.3]dodec-9-ene and dibromocarbene using density functional theory (DFT) B3LYP/6-31G*(d), *J Mol Model,* 21: 1610-2940.

Zeroual et al., 2015 – *Zeroual, A., El Haib, A., Benharref, A., El Hajbi A.* (2015). A combined experimental and theoretical study of highly chemioselectivity acetylation of diterpene, *Journal of Computational Methods in Molecular Design,* 5: 58-62.

Zeroual et al., 2015 – *Zeroual, A., Zoubir, M., Hammal, R., Benharref, A., El Hajbi, A.* (2015). Understanding the regioselectivity and reactivity of Friedel–Crafts benzoylation using Parr functions, *Mor. J. Chem*, 3: 356-360.

Zeroual et al., 2017 – *Zeroual A., El Idrissi M., El Ajlaoui R., Ourhriss N., Abouricha S., Mazoir N., Benharref A., El Hajbi A.* (2017). MEDT Study of the Mechanism and Regioselectivity of Diazocompounds and Alkenes in [3+2] Cycloaddition Reaction, *European Journal of Molecular Biotechnology*, 5(1), 43-49.

Zeroual et al., 2017 – *Zeroual A., El Idrissi M., Zoubir M., El Ajlaoui R., Abouricha S., El Hajbi A.* (2017). Theoretical Study of the Mechanism and Regioselectivity of Prop-2-Yn-1-Ol with Azide in [3+2] Cycloaddition Reactions, Open Access Journal of Translational Medicine & Research, 1(1), 1-5.

Zeroual et al., 2017 – *Zeroual A., Zoubir M., El Idrissi M., El ajlaoui R., El Haib A., Abouricha S., Mazoir N., El Hajbi A.* (2017). Theoretical Analysis of Reactivity and Regioselectivity in [1+2] Cycloaddtion Reaction of Some Monoterpenes with Dichlorocarbene, Global Journal of Science Frontier Research: B Chemistry, 17 (1) Version 1.0.

Zhang et al., 2015 – *Zhang B., White J. M., David J.* (2015). Regioselective synthesis of fullerene multiadducts via tether-directed 1,3-dipolar cycloaddition, *Org. Biomol. Chem.* 13, 10505-10510.

Zoubir et al., 2016 – *Zoubir M., Zeroual A., Benharref A., El Hajbi A.* (2016). Understanding the Holleman Rule in the Electrophilic Substitution Reaction Using Parr Functions, Journal of Computational Methods in Molecular Design, 6 (4), 1-4.

Zoubir et al., 2017 – *Zoubir M., Zeroual A., El Idrissi M., Bkiri F., Benharref A., Mazoir N., El Hajbi A.* (2017). Experimental and theoretical analysis of the reactivity and regioselectivity in esterification reactions of diterpenes (totaradiol, totaratriol, hinikione and totarolone), *Mediterranean Journal of Chemistry*, 6(4), 98-107.

Zoubir et al., 2017 – *Zoubir M., Zeroual A., El Idrissi M., El Haib A., Moumou M., Hammal R., Mazoir N., Benharref A., El Hajbi A.* (2017). Understanding the Chemoselectivity and Stereoselectivity in Michael Addition Reactions of β-Hydroxyparthenolides and Amines such as Pyrrolidine, Morpholine, Piperidine and 1-Methylpiperazine: a DFT Study, *Journal of Materials and Environmental Sciences*, 8 (3), 990-996.

Zoubir et al., 2017 – *Zoubir M., El Idrissi M., El Ajlaoui R., El Haib A., Abouricha S., Zeroual A., Benharref A., El Hajbi A.* (2017). Theoretical Investigation of the Chemo and the Regioselectivity of the Reaction Oxidation of Bicyclo[3.2.0]Hept-2-En-6-One by Hydrogen Peroxid, *European Reviews of Chemical Research,* 4(1), 28-33.

Published in the Slovak Republic
European Journal of Molecular Biotechnology
Has been issued since 2013.
ISSN: 2310-6255
E-ISSN: 2409-1332
2017, 5(2): 60-65

DOI: 10.13187/ejmb.2017.2.60
www.ejournal8.com

Extraction of Alkaloids from Three Nigerian Plants, Kola Acuminata (OJI IGBO) Vera Kola (OJI Hausa) and Garcinia Kola (BITTER KOLA)

I. A. A. Ibrahim [a, *], M. M. Mohammad [a], A. A. Faisal [a], H. Musa [b], G. Altine [c], A. Bununu [c]

[a] Department of Science Laboratory Technology, School of Science and Technology, Abubakar Tatari Ali Polytechnic Bauchi, Nigeria
[b] Department of Microbiology, School of Science, Bauchi State University, Gadau, Bauchi, Nigeria
[c] Department of Agricultural Technology, College of Agriculture Bauchi, Nigeria

Abstract

The extraction of alkaloid from Garcinia Kola (bitter kola) Kola Accuminata (Oji Igbo) and Kola Vera (Oji Hausa) were carried out using 10 % enthanoic acid and 10 % methanol to separate the alkaloid from the residue. The alkaloid was extracted using ammonium hydroxide from the sample results obtained on alkaloid for the sample 9.16 % Oji Igbo, 6.20 % Oji Hausa and 8.20 % Bitter Kola. It was observed that the percentage of alkaloids was highest in Oji Igbo and the least for Oji Hausa. The extracts all exhibited antimicrobial activity against he tested organisms. These activities decreasing with a decrease in extract concentrations. The results suggest the usefulness of thee nuts in the treatment or management of microbial infections without the dangers of side effects from synthetic drugs.

Keywords: Extraction, Alkaloids, Kola Acuminata, Garcinia Kola.

1. Introduction

Kola nut is a plant that is grown in the costal rain forest in south western and south eastern part of Nigeria bitter Kola which is known as (Garcinia kola) is an African wonder nut (Wakeel et al., 2004; Leke, 2015). It comes from Garcinia Kola forest which belongs to the family of clusiaceae (Momoh Johnon, 2014; Leke, 2015).

Traditionally these nuts were chewed as a masticatory substance, to stimulate the flow of saliva (D.O. et al., 2014). But are widely consume as snack in West and Central Africa. The kernels of the nuts are widely traded and eaten as a stimulant (D.O. et al., 2014; Omwirhiren et al., 2016).

Medicinal plants are plants that are used to cure different types of disease e.g Garcinia Kola (Bitter kola) (Musa et al., 2011; Eltayeb et al., 2018). It cleans the digestive system without any side effect like abdominal problems alern when a lot of the nuts are eaten (Eleazu et al., 2012; Rajeshwar et al., 2016; Eltayeb et al., 2016).

Medicinal plants have been identified and used throughout human history. The plant exhibits very potent pharmacological activities such as antioxidants, antibacterial, antiviral, anti-fungal and anti-inflammatory properties (Akinpelu, 1999; Leke, 2015). The nuts are commonly used in Nigeria

* Corresponding author
E-mail addresses: iliyasuibrahim@gmail.com (I.A.A. Ibrahim)

as well as other countries for the treatments of common microbial infections (Eleazu et al., 2012; Patil, Gaikwad, 2011; Qadir et al., 2015).

This work is mainly designed to extract alkaloids from kola nuts commonly used in Nigeria (Garcinia Kola)

2. Material and methods

Sample collection and preparation

Sample collection

Garcinia kola (bitter kola) and vera kola (Oji Hausa) were bought at wuntin-dada, market while Kola Accuminata (Oji Igbo) was bought at central market Bauchi in Bauchi State, Nigeria.

Sample preparation

The sample was peeled and sliced into very small sizes and dried at a room temperature for 5days to constant weight. It was then grinded into fine powder and stored in an air tight plastic rubber container ready for extraction

Extraction method

Methanol extraction:

10g of each of the sample was poured into a conical flask and 100 $cm^3$of methanol into the sample and covered and vigorously shake. It was then kept for 72 hours and filtered and ready for the analysis.

Extraction of Alkaloid

Ethanol, ammonium hydroxide and acetic acid were used. 5 g of the sample was weighed into a 250ml beaker and 100ml of 10 % acetic in ethanol was added and covered. This was vigorously shaken, venting the mounted pressure and allowed to partition into organic and aqueous layers for 4hours. This was filtered and the extract was concentrated on a water bath to one quarter of the volume.

Concentrated ammonium hydroxide was added drop wise to the extract until the precipitate was complete. The ammonium (NH_3) was added to neutralize the acid (to make alkaline), the whole solution was allowed to settle and the precipitated was collected, and washed with dilute (NH_4OH) ammonium hydroxide and the filtered residue was the alkaloid which was dried and weighed. The organic layer was tested for alkaloid with mayers and wagner's reagents respectively to ensure full extraction.

Phytochemical analysis

Test for tannins

In this case 1 g of the sample powder was boiled with 5.0ml of water, filtered and used for the test.

Test for saponins

20 ml of distilled water was added to 0.25 g of the sample powder in a test tube and boiled gently in a hot water bath for 20 minutes. The mixture was filtered hot and allowed to cool. The filtrate was used for the following tests.

1. **Frothing test:** – 5 ml of the filtrate was diluted with 20ml of distilled water and vigorously shaken and left to stand, stable from was observed in the filtrated which indicate the presence of saponins.
2. **Emulsion test:** – 2dropolive oil was added to the following solution and the content shaken vigorously. An emulsion was formed from the frothing solution which showed the presence of saponins
3. **Fehling's test:** – 5 ml of Fehling's solution A and B was added to 5 ml of the filtrate and the content was heated in a water bath. A reddish precipitate which turned brick red on further heating with added sulphuric acid which indicate the presence of saponins.

Anti-microbial activities

The agar-well diffusion method was used to determine the anti-microbial activity of the extracts. The bacterial isolate was first grown in nutrient broth for 18hrs. 0.2ml of the log phase culture was aseptically used to seed a molten nutrient agar which had been cooled to about 45°C,

mixed gently and poured into steric petri dishes and allowed to set. Extract was tested at 50mg/ml concentration this was delivered into walls (5mm in diameter) bored into surface of the already seeded nutrient agar plates.

Standard antibiotic concentration of ciprofloxacin 25mg/l, amoxicillin 25mg/l and fluconazole 5mg/l was assayed using the agar well diffusion techniques. The fungal isolate was treated in a sabouroud dextrose broth before were assayed using sabouroud dextrose agar. The bacterial plates were incubated at 37^{o}C for 24hours, while the fungal plates were incubated at 25^{o}C for 72hours. The zones of inhibition was measured in mm diameter and recorded.

Determination of minimum inhibitory concentration (MICs)

The ager dilution method was also used (Russell and Fur, 1977). The extract was incorporated into molten nutrient ager at concentrations of 40,30,20,15,10,7.5 and 5.0, 2.5mg/ml aseptically, mixed gentle in sterile petri dishes and then allowed to set. The surface of the agar plate was allowed to dry properly before inoculating with appropriate bacterial and fungal culture previously diluted to about 10^{6}cfu/ml. the plate was then incubated at 37^{o}c for up to 72hours after about 30 minutes of inoculation. The lowest concentration preventing visible growth in each determination was taken as the maximum inhibitory concentration. The mean of the three replicate determinations was obtained.

3. Results and discussion

Table 1. Phytochemical analysis of Garcinia kola (Bitter kola)

Metabolite	Methanolic extract
Saponin	+
Tannin	+

Key:
+ = Present
- = Absent

Table 2. Phytochemical analysis of Kola accuminata (Oji Igbo)

Metabolite	Methanolic extract
Saponin	+
Tannin	+

Key:
+ = Present
- = Absent

Table 3. Phytochemical analysis of Kola vera (Oji Hausa)

Metabolite	Methanolic extract
Saponin	+
Tannin	+

Key:
+ = Present
- = Absent

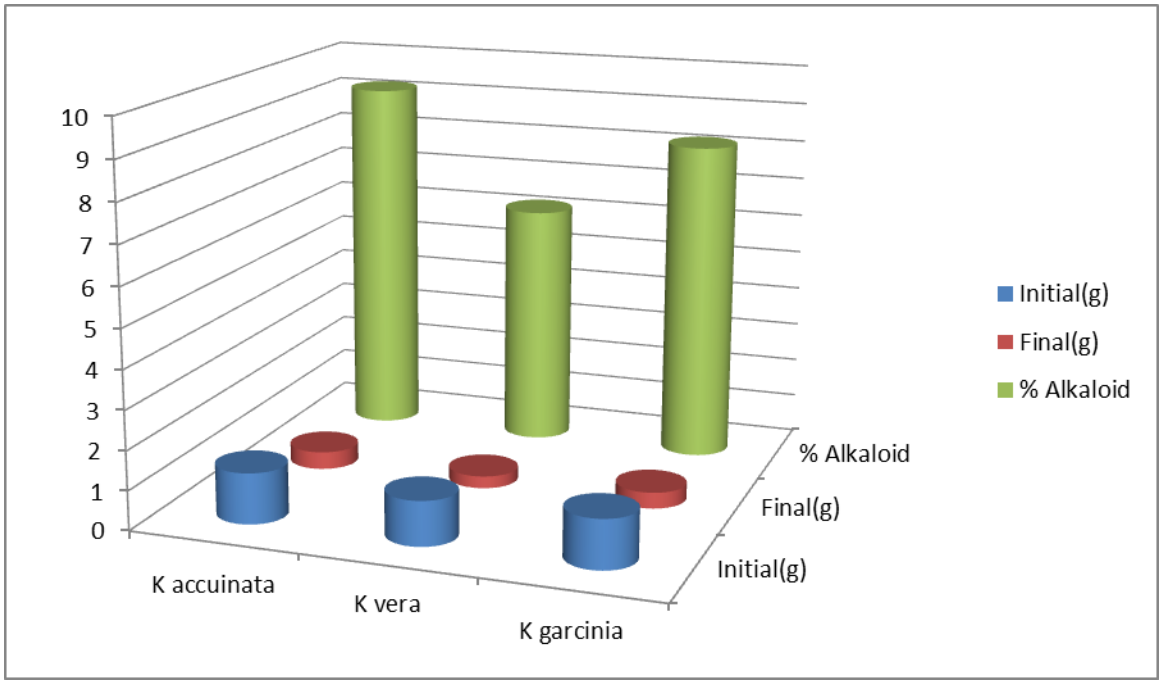


Fig. 1. Percentage Alkaloid in Kola nuts

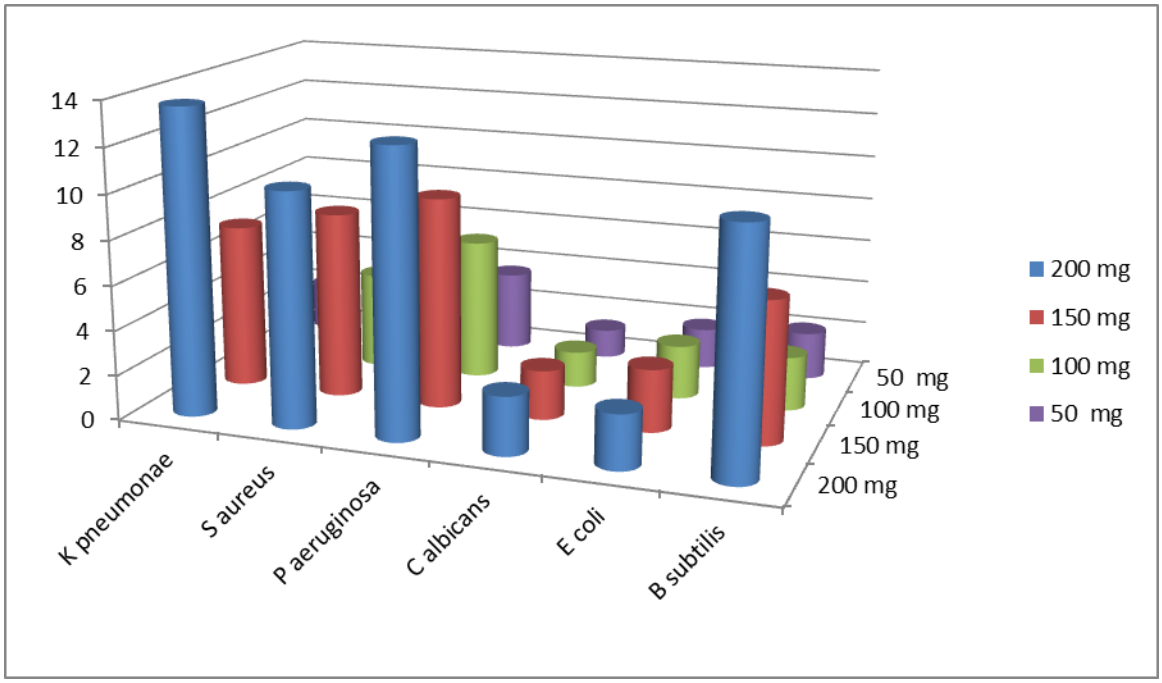


Fig. 2. Zone of inhibition (mm) of Garcinia Kola extract against the test organisms at different concentrations of extract

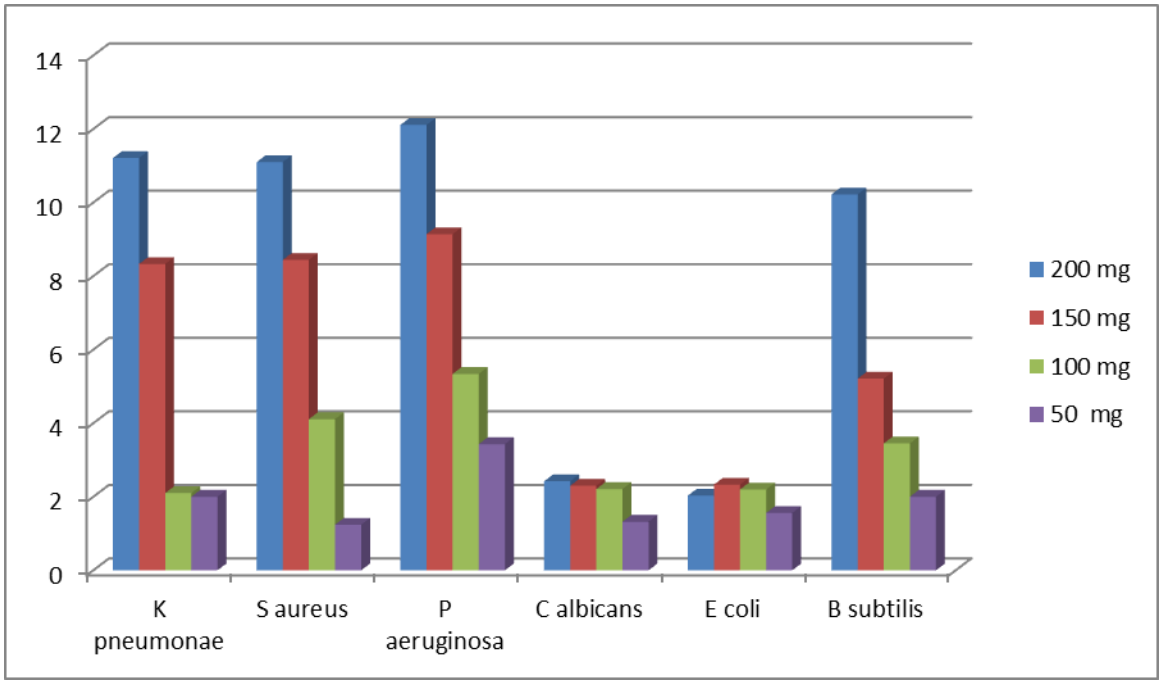


Fig. 3. Zone of inhibition (mm) of Vera Kola extract against the test organisms at different concentrations of extract

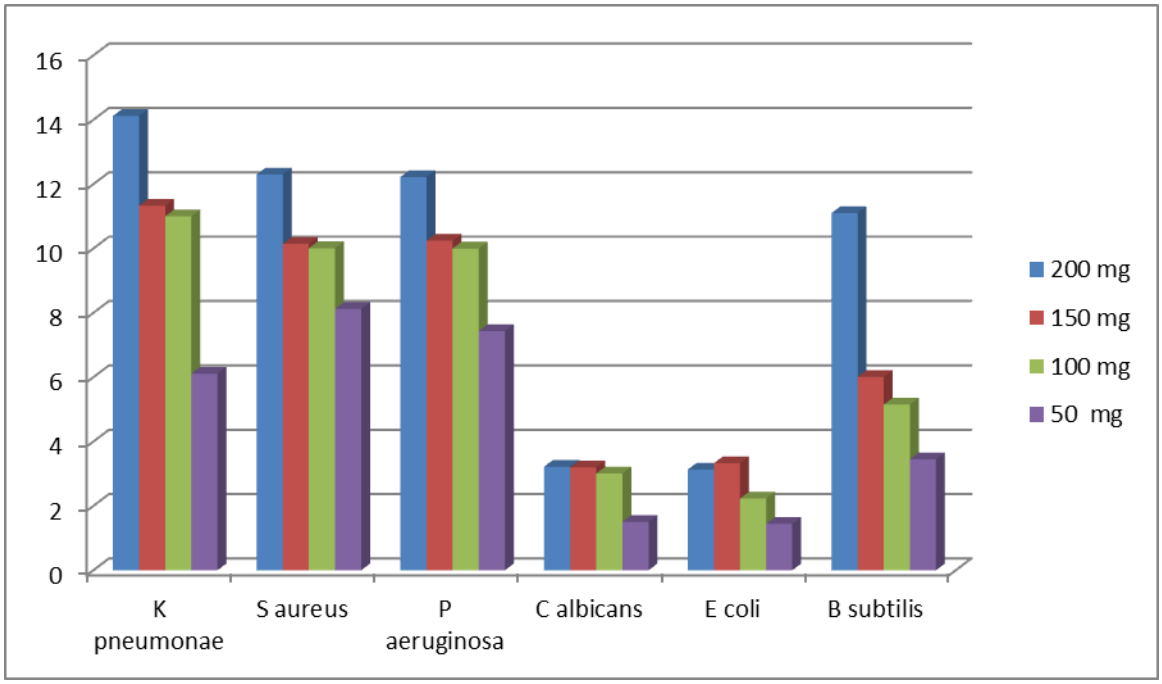


Fig. 4. Zone of inhibition (mm) of Kola acuminate extract against the test organisms at different concentrations of extract

3. Discussion

The phytochemical screening tests on the aqueous extract of Garcinia Kola, kola vera and kola accuminata seed showed that it had the presence of the secondary metabolites tested for (Table 1) which is also reported other researchers (Gumgumjee, Hajar, 2012; Bekele, 2015; Omwirhiren et al., 2016; Rajeshwar et al., 2016). Zones of inhibition of the tested organisms showed that the three tested nuts exhibited a myriad of zone of inhibitions on the tested organisms in response to varying degree of concentrations of the extracts (Fig. 2, 3 and 4). This expected when the concerntrations are altered (Wakeel et al., 2004; Eleazu et al., 2012; Leke, 2015) Diethyl ether

extract of Garcinia Kola, vera Kola and Kola acuminate seed are similarly strongly active against Pseudomonas aeruginosa; Bacillus subtilis and klebsiella pneumonia with similar zones of inhibition (Fig. 2, 3 and 4). The 50mg concentrations showed moderate activity against Klebsiella pneumonia and Bacillus subtilis respectively and poor inhibitory activity against Pseudomonas aeruginosa and Staphylococos aureus respectively.

All three concentrations of the diethyl ether extract of Garcinia Kola seed, Kola vera seed and kola accuminata showed poor inhibitory action against candida albicans in the order, the activity of 100mg>150mg>100mg >50mg with decreasing zones of inhibition respectively (Fig. 2, 3 and 4).

References

Akinpelu, 1999 – *Akinpelu, D. A.* (1999). Antimicrobial activity of Vernonia amygdalina leaves. *Fitoterapia*, *70*(4), 432–434. https://doi.org/10.1016/S0367-326X(99)00061-1

Bekele, 2015 – *Bekele, T.* (2015). Invivo Anti-Malarial Evaluation of Leaf Extract of Vernonia amygdalina Del. (Asteraceae) against Plasmodium berghei A Thesis submitted to Addis Ababa University, School of Graduate Studies , and Department of Biochemistry in Partial fulfillment of the.

D.O. et al., 2014 – *D.O., A., K.T., O., T.P., O., O.J., O., & I.A, A.* (2014). Nutritional, Minerals and Phytochemicals composition of Garcinia cola [Bitter cola] and Aframomum melegueta [Alligator pepper]. *Journal Of Environmental Science, Toxicology And Food Technology*, *8*(1), 8 6-91.

Eleazu et al., 2012 – *Eleazu, C. O., Awa, K. C., & Chukwuma, E.* (2012). Comparative study of the phytochemical composition of the leaves of five Nigerian medicinal plants. *E3 Journal of Biotechnology and Pharmaceutical Research*, *3*(2), 42–46.

Eltayeb et al., 2016 – *Eltayeb, I. M., Abdel, H., Ali, R., & Muddather, A. K.* (2016). Madurella mycetomatis Susceptibility to Anogeissus leiocarpus Stem Bark Extracts. *American Journal of Research Communication*, *4*(3), 68–86.

Eltayeb et al., 2017 – *Eltayeb, I. M., Abdel, H., Ali, R., & Muddather, A. K.* (2017). Antioxidant Activity and Cytotoxic Studies of Anogeissus leiocarpous Root, Leaf and Stem. *American Journal of Research Communication*, *4*(3), 52–67.

Gumgumjee, Hajar, 2012 – *Gumgumjee, N. M., & Hajar, A. S.* (2012). Antimicrobial efficacy of Casuarina equisetifolia extracts against some pathogenic microorganisms. *Journal of Medicinal Plants Research*, *6*(47), 5819–5825. https://doi.org/10.5897/JMPR12.741

Leke, 2015 – *Leke, O.* (2015). Medical plants and natural products chemistry in Achebe's "things fall apart": The metaphor of kola. *International Journal of Arts and Humanities*, *3*(3), 70–76.

Momoh Johnon, 2014 – *Momoh Johnon, A. S. M. and G. K.* (2014). sjbt-194. *Science Journal of Biotechnology*, *1*(1), 1–7.

Musa et al., 2011 – *Musa, M. S., Abdelrasool, F. E., Elsheikh, E. A., Ahmed, L. A. M. N., Mahmoud, A. L. E., & Yagi, S. M.* (2011). Ethnobotanical study of medicinal plants in the Blue Nile State, South-eastern Sudan. *Journal of Medicinal Plants Research*, *5*(17), 4287–4297.

Omwirhiren et al., 2016 – *Omwirhiren, E. M., James, S. A., & Abass, A. O.* (2016). Saudi Journal of Medical and Pharmaceutical Sciences The Phytochemical Properties and Antimicrobial Potentials of Aqueous and Methanolic seed Extract of Cola nitida (Vent.) and Cola acuminata (Beauvoir) grown in South West, Nigeria. *Saudi Journal of Nedical and Pharmaceutical Sciences*, 354–363. https://doi.org/10.21276/sjmps.2016.2.12.6

Patil, Gaikwad, 2011 – *Patil, U. H., & Gaikwad, D. K.* (2011). Ethno-pharmacological Review of a Herbal Drug : Anogeissus latifolia. *International Journal of Pharma Sciences and Research*, *2*(1), 41–43.

Qadir et al., 2015 – *Qadir, U., Paul, V. I., & Ganesh, P.* (2015). Preliminary phytochemical screening and in vitro antibacterial activity of Anamirta cocculus (Linn.) seeds. *Journal of King Saud University - Science*, *27*(2), 97–104. https://doi.org/10.1016/j.jksus.2014.04.004

Rajeshwar et al., 2016 – *Rajeshwar, Y., Sreekanth, T., Kahsay, G., Hussen, D. A., & Eticha, T.* (2016). Hepatoprotective activity of Anogeissus latifolia bark in ethanol intoxicated rats. *The American Journal of Science and Medical Research*, *1*(2), 198–201. https://doi.org/10.17812/ajsmr1210.Published

Wakeel et al., 2004 – *Wakeel, O. K., Aziba, P. I., Ashorobi, R. B., Aderibigbe, A., & Awe, E. O.* (2004). Neuropharmacological Activities of Ficus Platyphylla Stem Bark in Mice, *7*, 75–78.

Published in the Slovak Republic
European Journal of Molecular Biotechnology
Has been issued since 2013.
ISSN: 2310-6255
E-ISSN: 2409-1332
2017, 5(2): 66-73

DOI: 10.13187/ejmb.2017.2.66
www.ejournal8.com

The QseB/QseC Signaling Affects Initiation of Chromosomal Replication through Regulating Expression of the DnaA Protein in *E.coli*

Baigalmaa Luvsandorj [a, *], Morigen [b]

[a] Institute of General and Experimental Biology, Mongolian Academy of Sciences (MAS), Ulaanbaatar, Mongolia
[b] School of Life Science, Inner Mongolian University (IMU), Hohhot, Inner Mongolia, China

Abstract

The *Escherichia coli* two-component system, QseB/QseC signaling regulates expressions of more than 50 genes encoding flagellar proteins and proteins associated with virulence. In this work, we found that absence of the *qseB* gene led to an early initiation of chromosomal replication in a manner of medium-independent, the early initiation was shown to be reversed by ectopic expression of QseB from a plasmid p*qseB*. Further, absence of the *qseB* gene resulted in an increase of DnaA concentration and the purified QseB protein did not interact with the *oriC* DNA *in vitro*. The early initiation was also found in the *ΔqseC* mutant cells. These results indicate that absence of the QseB/QseC signaling increases concentration of DnaA and the later subsequently triggers the early initiation of replication. Flourescene microscope analysis showed that the QseC protein localized at cell membrane while QseB was in cytosol.

Keywords: QseB/QseC signaling, QseB protein, initiation of DNA replication, DnaA protein, *E. coli*.

1. Introduction

Quorum sensing is via the production of compounds called as autoinducers that involved in pathogenesis and nonpathogenic, including flagellation, motility, shiga toxin production, cell growth (Sperandio et al., 2001; Liu et al., 1995) and two-component signaling system (Sharma et al., 2010; Kostakioti et al., 2009: Sperandio et al., 2004, 2003); as well as genes involved in bacterial metabolism, nucleotide and protein biosynthesis, DNA repair, cell growth division and DNA replication (Matthew et al., 2006; Helen et al., 1998) in *E.coli*.

The QseB/QseC (Quorum sensing *E. coli* regulators B and C) signaling is responsible for cell-to-cell communication, quorum sensing, controlling bacterial behavior by various signal molecules. QseC is a transmembrane protein that interacts through its periplasmic sensory domain with AI-3 and the stress hormones epinephrine and norepinephrine to undergo autophosphorylation at its cytoplasmic domain (Hughes et al., 2009; Marcie et al., 2005). The phosphorylated QseC transfers the phosphate to its cognate response regulator QseB, which activates transcription of FlhDC, the master transcriptional regulator of the flagellar gene complex, and autoregulates its own

* Corresponding author
E-mail addresses: baigalmaaluvsandorj@gmail.com (B. Luvsandorj)

transcription by binding to the *flhDC* and QseBC promoters, respectively (Clarke et al., 2005; Kendall et al., 2007).

Few proteins participated in the initiation of chromosomal replication process (Zyskind and Smith, 1986) including DnaA (initiator), DnaB (helicase), DnaC (laoder) and DnaG (primase). In the first event of initiation, several DnaA - ATP molecules bind to specific 9 bp sites in *oriC* (Funnell et al., 1987; Fuller et al., 1984). An architectural protein IHF binds *oriC* and bends the DNA to help the interaction between the DnaA proteins and the AT rich region (Messer, 2002; Hwang and Kornberg, 1992), and DNA strands is separated at the AT rich region. (Messer, 2002; Hwang and Kornberg, 1992), and DNA strands is separated at the AT rich region. The DnaB/DnaC complex interacts with an open complex, DnaC loader loads a DnaB helicase onto each of the single strands generated by the melting process (Funnel et al., 1987). DnaC leaves the complex, the DnaB helicase lengthenes the single stranded region and then loads DnaG primase, which synthesizes RNA primer. Finally, the DNA polymerase III holoenzyme is the single stranded DNA binding (SSB) protein and DNA gyrase are loaded (Morigen et al., 2005; 2003).

We investigated the influence of the QseB/QseC signaling on initiation of DNA replication, and its localization on cell.

2. Materials and Methods

Bacterial Strains and Plasmids

All bacterial strains used were K12 listed in Table 1. PCR fragment of the aspC gene with its native promoter region using a pair of primers of 5′ GCGGATCCCCAGTCTTTATCGACTTCACCC 3′ and 5 ′CGAAGCTTGATTAGCGTCAGCCTGACGCGCAG 3′ was inserted into plasmid pACYC177 at BamHI and HindIII sites, resulting in a plasmid over-expresssing QseB, namely pACYC177- *qseB*. The plasmid was introduced into competent cells by eletroporation. Growth Media and Condition Cells were grown in LB or ABTGcasa medium (Morigen et al., 2005) at 37 °C. 50 mg/ml of kanamycin, 30 mg/ml of chloramphenicol and 50 mg/ml of ampicillin were added when required for selection.

Table 1. Strains and plasmids used in this study

Strains	Relevant genotype	Reference
BW25113	Wild type	Baba *et al* (2006)
MOR318	*BW25113/qseB::Kan^R*	Yixin.Shi (2011)
B1	*BW25113 /pACYC177-qseB*	This work
B2	*MOR318 /pACYC177-qseB*	This work
MOR578	*BW25113 /qseC::Kan^R*	Yixin.Shi (2013)
MOR541	*DH5a GFP-qseC*	Yixin.Shi (2012)
B3	*MOR578 / GFP qseC*	This work
B4	*MOR318 / GFP qseC*	This work
MOR611	*DH5a GFP-qseB*	Yixin.Shi (2013)
B5	*MOR318 / GFP qseB*	This work
B6	*MOR578 / GFP qseB*	This work
Plasmids		
pACYC177	repp15AApR (bla) KmR	Chang (1978)
pMD™19	Cloning vector	Takara
pET-28a	*repColE1 neo lacI PT7*	EMD Biosciences
p*qseB*	*qseB* gene on pACYC177	this work
pCA24N	rep pMB1CmRlacIqppT5-lacthisGFP	Kitagawa (2006)
pQseB-GFP	*qseB* gene on pCA24N (pCA24N derivative)	Kitagawa (2006)
pQseC-GFP	*qseC* gene on pCA24N (pCA24N derivative)	Kitagawa (2006)

Flow Cytometry Analysis

Exponentially growing cells in LB, ABTGcasa medium were collected at OD600/450= 0.15, and then treated with 300 mg/ mL of rifampicin and 10 mg/mL of cephalexin for 3–5 generations. Rifampicin inhibits initiation of replication through preventing transcription which is required for replication initiation but allows ongoing rounds of replication to finish, whereas cephalexin blocks cell division (Boye et al., 1999; 1986). After treatment with these drugs, cells end up with an integral number of chromosomes, representing the number of origins per cell at the time of drug addition (Skarstad et al., 1986). Cells treated with rifampicin and cephalexin were fixed in 70 % ethanol. Slowly growing cells in medium of ABTGcasa were collected by centrifugation at OD450= 0.15 and fixed in 70 % ethanol. Following one wash in Tris-HCl buffer (pH7.5), cells were stained in Hoechst33258 for 30 min, and analyzed by flow cytometer (BD LSRFortessa). Preparation of standard sample and post analysis were as described previously (Morigen et al., 2005; Wold et al., 1994).

Determination of Total Protein per Cell

Exponentially growing cells in ABTGcasa at 37 ^{o}C were collected on ice. 9 mL of the cell culture were harvested by centrifugation at 4 ^{o}C, washed in 1 ml of TE buffer, and resuspended in 200 ml TE buffer containing 1 % SDS and glycerol and then boiled for 5 min. Total protein amount in a fixed volume of the cell extract mentioned above was determined by a colorimetric assay (BCA kit, pierce) as described previously (Morigen et al., 2003). To measure the number of cells in a certain volume of the cell culture, 10 mL of the cells mentioned above were diluted 104 and 105 times and then plated on LB agar plates with required antibiotics. After incubation at 37 ^{o}C overnight, the number of the colonies were counted. Using the amount of protein in a certain volume of cell extract from 9 ml of the culture and the number of cells in 10 mL of the culture, the protein amount per cell was calculated.

Western Blotting

The cell extract mentioned above was also used to determine the DnaA concentration by Western blotting. Fixed amounts of cell extracts were subjected to SDS-PAGE (12 %). The protein was transferred to a polywinylidene difluoride (PVDF) membrane by semi-dry blotting. The membrane was probed with anti-rabbit antibody for DnaA and secondary antibody which was also antirabbit IgG (TransGen Biotec) as described previously (Morigen et al., 2001).

3. Results

1. The QseB protein does not directly interacts with oriC region

Our previously study resulted shown that the absence of QseB/QseC signaling leads to early initiation of DNA replication but mechanism behind is elusive (Fig. 1) (Baigalmaa, Morigen, 2016). It could be possible the QseB protein may interact with *oriC* region to assist initiation of replication at *oriC*.

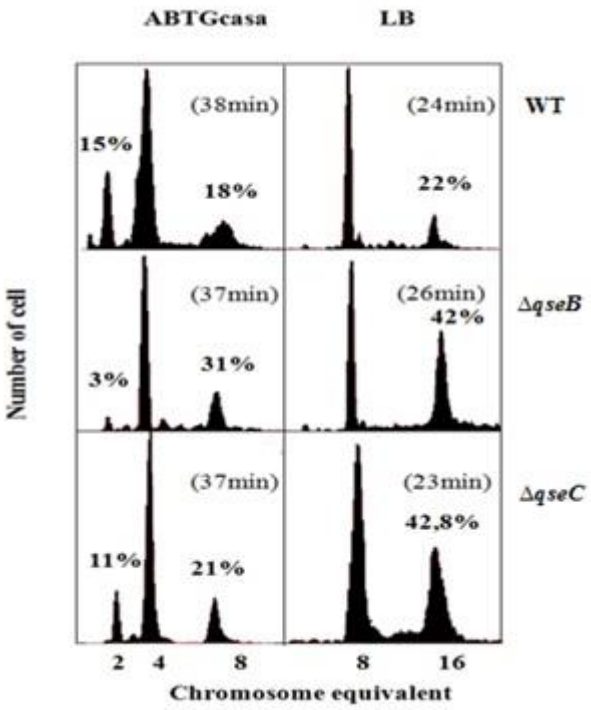


Fig. 1. Absence of QseB/QseC signaling results in early initiation of replication. Exponentially growing cells in media indicated were treated with rifampicin and cephalexin for 3-5 generations, and fixed in 70 % ethanol. Cells were analyzed by Flow cytometer after staining in Hoechst 33258 for 30 min. Y-axis represents number of cells measured while X-axis indicates chromosome equivalents per cell. 10000 cells were included in each measurement, other information are indicated

To test the possibility, we purified the QseB-His protein. For purification, the *E. coli* strain containing pET28a-*qseB* was grown at 37 °C in LB to an OD_{600} of 0.6, at which point arabinose was added to a final concentration of 0.2 % and allowed to induce for three hours. Protein purification was then performed using nickel columns according to manufacturer's instructions (Qiagen). The purifed protein was checked on an SDS-PAGE gel (Fig. 2).

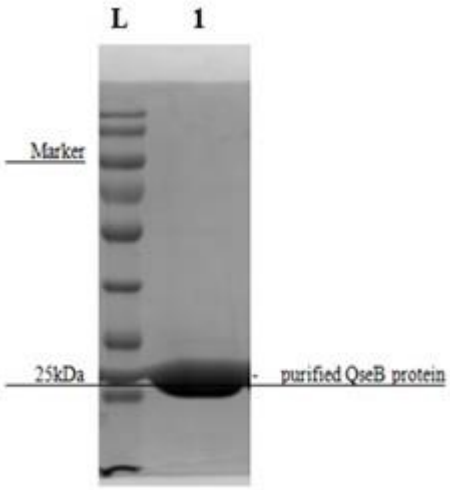


Fig. 2. Purification of the QseB protein. Purified the QseB-His protein and test the production with SDS-PAGE. L represents protein ladder; while 1 represent the QseB protein, whose molecular weight is 25kDa

To test interaction between the QseB protein and *oriC*, the QseB protein was incubated with the *oriC* DNA (PCR amplified fragment, about 1.1 kb), and then loaded on agarose gel for gel-shift assay. When the *oriC* DNA is bound with QseB, the *oriC* fragment would migrate slower than one that is not bound with the protein in gel-shift assay. As shown in fig. 3, the *oriC* DNA was not found to be shifted. The BSA protein was included in the experiment as a negative control. The result suggests that QseB does not directly interact with the *oriC* region.

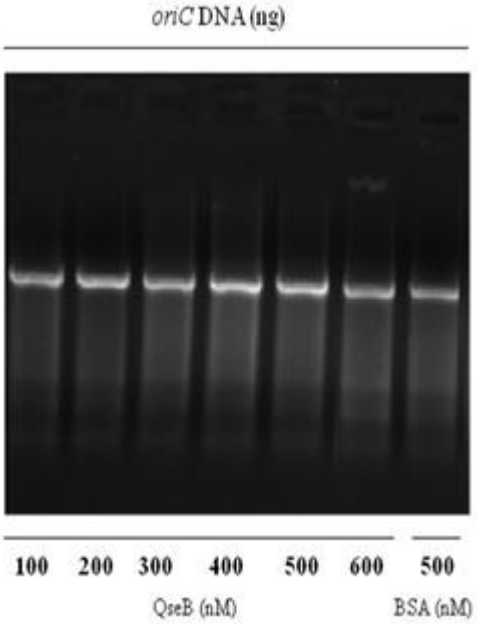


Fig. 3. The QseB protein does not interacts with *oriC*

For Gel shift assay, the *oriC* DNA was incubated with QseB at different concentrations as indicated at bottom. Then the proposed complex was loaded on agarose gel and analyzed be gel-shift assay. The gel was then stained in Safe-green nucleic acid dye. The BSA protein was included in the experiment as a negative control.

2. *Absence of QseB enhances concentration of the DnaA protein*

In the previous section, we showed that QseB did not interact with the *oriC* region, then how does the QseB protein affect initiation of replication? It could be possible that the QseB/QseC signaling would change concentration of the DnaA protein. The DnaA protein is is essential for initiation of replication at the *Escherichia coli oriC* both *in vivo* (Tomizawa and Selzer, 1979) and *in vitro* (Fuller et al., 1984). To check the possibility of changes in DnaA concentration, western blotting technique was used to detect concentration of the DnaA protein (Fig. 4) as described previously (Liu et al., 2014). Interestingly, concentration of the DnaA protein in *ΔqseB* mutant was increased significantly relative to that of wild-type (WT) cells (Fig. 4) as we expected. The results indicate that the QseB protein affects initiation of replication through regulating expression of the DnaA protein since DnaA directly initiates replication by interacting with the *oriC* DNA.

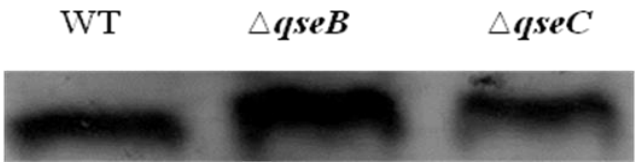


Fig. 4. The QseB protein enhances the DnaA protein amount per cell

Absence of the QseB protein enhances concentration of the DnaA protein. The DnaA protein concentration was determined by Western blotting as described previously (Liu et al., 2014).

3. The QseC protein localizes at cell membrane while QseB in cytosol

As mentioned above, the sensor protein, QseC, localizes at cell membrane. To detect subcellular localization of the QseB protein. The *ΔqseB*/pQseB-GFP and *ΔqseC*/pQseC-GFP cells were exponentially grown in ABTGcasa medium with induction of QseB-GFP and pQseC-GFP fusion by IPTG. Cells were collected, fixed in 70% ethanol and then visualized under fluorescent microscope. We found that the QseC-GFP protein was clearly on membrane while QseB-GFP was at two poles in some cells or all over in cytosol (Fig. 5). The results confirm that QseC-GFP is on membrane while QseB-GFP is cytosol.

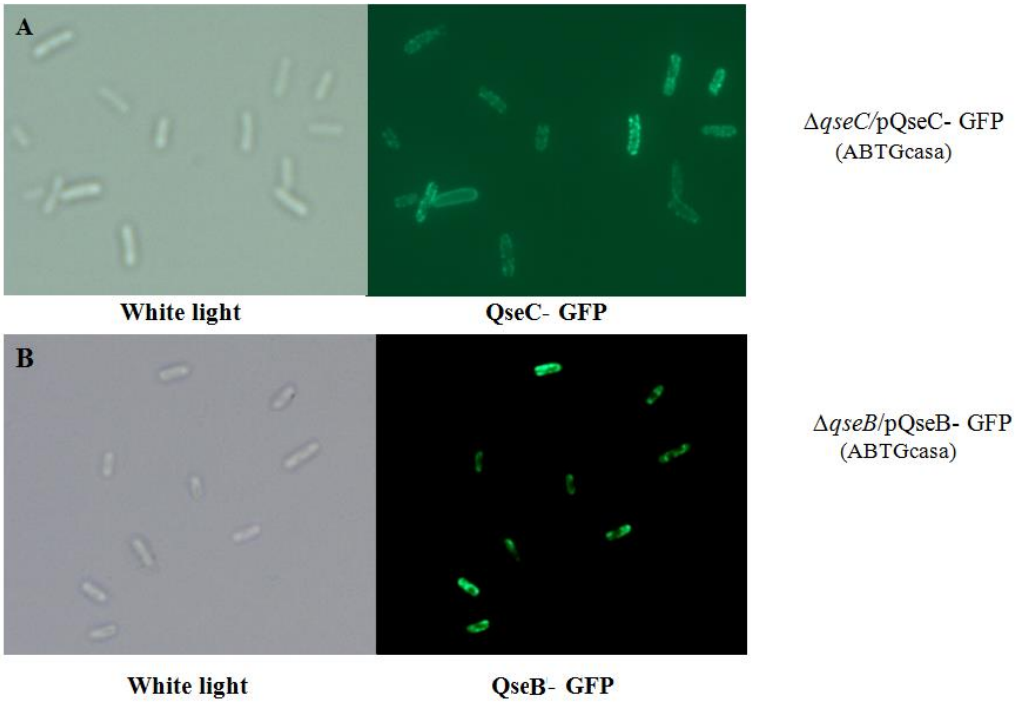


Fig. 5. The QseC protein localizes at cell membrane while QseB in cytosol. Exponentially growing cells were fixed in 70 % ethanol and then visualized by fluorescent microscope. The QseC- GFP (A) or QseB- GFP fusion (B) is in green

4. Conclusion

The Quorum-sensing QseB/QseC two-component pathway regulates bacterial motility in response to bacterially created quorum-sensing signals called autoinducers (AI) and the human hormone, epinephrine (Sperandio et al., 2005; Sperandio et al., 2002). Here we showed that the QseB/QseC signaling influenceed intiation of chromosomal replication.

The DnaA protein exerts a positive regulation on initiation of replication (Løbner-Olesen et al., 1989; Braun et al., 1985). The amount or concentration of DnaA protein is thought to be a limiting factor for initiation of replication. We showed that the absence of both *qseB* and *qseC* genes led to the early initiation of chromosomal replication (Fig. 1), indicating that QseB/QseC signaling indeed affects initiation of replication. To understand the mechanism behind, the purified QseB protein (Fig. 2) was incubated with the *oriC* DNA fragment, but no interaction between QseB and *oriC* was found (Fig. 3). Interestingly, however, DnaA concentration was dramatically increased in the absence of *qseB* gene (Fig. 4). The result can explain why initation occurs early in Δ*qseB* cells since the increased initiator protein DnaA concentration might change the timing of replication initiation. In accordance with the observation, initiation of replication was found to be affected by changing the amount of DnaA per cell in *aspC* mutant which is defective in AspC-mediated metabolism of aspartate (Liu et al., 2014). The sensor protein, QseC, localizes at cell membrane. To detect subcellular localization of the QseB protein (Fig. 5).

5. Acknowledgments

We are deeply grateful to China Scholarship program and Life Science College of Inner Mongolia University for their support of our study.

References

Baigalmaa, Morigen, 2016 – *Baigalmaa L, and Morigen* (2016). The Quorum sensing *Escherichia coli* regulators B and C (QseB /QseC) signaling acts at initiation of DNA replication. *Scientific Journal of Mongolian University of Science and Technology* with index ISSN 1560-8794, (2016). N19/183

Boye, Løbner-Olesen, 1991 – *Boye E, Løbner-Olesen A* (1991). Bacterial growth control studied by flow cytometry. *Res Microbiol.* 142: 131–135.

Braun et al., 1985 – *Braun RE, O'Day K, Wright A* (1985). Autoregulation of the DNA replication gene dnaA. *E. coli.* K-12. Cell 40: 159–169. 33.

Clarke, Sperandio, 2005 – *Clarke MB, Sperandio V.* (2005). Transcriptional regulation of flhDC by QseBC and sigma (FliA) in enterohaemorrhagic. *Escherichia coli. Mol. Microbiol.* 57**:**1734–1749.

Feng et al., 2014 – *Feng Liu, Qimuge, Jianfeng Hao, Huijuan Yan, Trond Bach, Lifei Fan and Morigen* (2014). AspC-Mediated Aspartate Metabolism Coordinates the *Escherichia coli. Cell Cycle PLoS ONE*, 9(3): p. e92229.

Fuller et al., 1984 – *Fuller RS, Funnell BE and Kornberg A.* (1984). The dnaA protein complex with the *Escherichia coli* chromosomal replication origin (oriC) and other DNA sites. *Cell,* 38(3): p. 889-900.

Funnell et al., 1987 – *Funnell BE, Baker TA and Kornberg A.* (1987). In vitro assembly of a prepriming complex at the origin of the *Escherichia coli* chromosome. Jounal of Biol Chem, 262(21): p. 10327-34.

Kendall et al., 2007 – Kendall, M. M, Rasko, D. A and Sperandio, V. (2007). Global effects of the cell-to-cell signaling molecules autoinducer-2, autoinducer-3, and epinephrine in a luxS mutant of *enterohemorrhagic Escherichia coli.* Infect. Immun. 75:4875-4884

Kostakioti et al., 2009 – *Kostakioti M, Hadjifrangiskou M, Pinkner J S and Hultgren SJ.* (2009). QseC mediated dephosphorylation of QseB is required for expression of genes associated with virulence in uropathogenic Escherichia coli. *Journal of Mol. Microbiol*, 73: p. 1020–1031

Withers, Nordstrom, 1998 – Helen L. Withers and Kurt Nordstrom. (1998). Quorum-sensing acts at initiation of chromosomal replication in *Escherichia coli. Proc. Natl. Acad Sci USA Microbiol,* 95: p. 15694–15699.

Hughes et al., 2009 – Hughes DT, Clarke MB, Yamamoto K, Rasko DA, and Sperandio V. (2009). The QseC adrenergic signaling cascade in *enterohemorrhagic E. coli* (EHEC). PLoS Pathog Acad Sci USA. 5**,** e1000553

Hwang, Kornberg, 1992 – Hwang D S, Kornberg A. (1992). Opening of the replication origin of *Escherichia* coli by DnaA protein with protein HU or IHF. *J Biol Chem*, 267(32): p. 23083-6.

Liu, Matsumura, 1994 – Liu X, Matsumura P. (1994). The FlhD/FlhC complex, a transcriptional activator of the *Escherichia coli* flagellar class II operons. *Journal of Bacteriol*, 176: p. 7345–7351.

Løbner-Olesen et al., 1989 – *Løbner-Olesen A, Skarstad K, Hansen FG, von Meyenburg K, Boye E* (1989). The DnaA protein determines the initiation mass of Escherichia coli K-12. *Cell*, 57: 881–889

Matthew, Sperandio, 2006 – *Matthew W and Sperandio V.* (2006). Autoinducer 3 and epinephrine signaling in the kinetics of locus of enterocyte effacement gene expression in enterohemorrhagic Escherichia coli. *Journal of Infect Immun*, 74: p. 5445-55.

Matthew et al., 2006 – Matthew W, Sircili Marcelo P and Sperandio V (2006). AI-3 synthesis is not dependent on luxS in *Escherichia coli. J Bacteriol*, 188: p. 5668-81.

Matthew, Sperandio, 2006 – Matthew W and Sperandio V (2006). Quorum sensing in *Escherichia coli* and *Salmonella. Int. Journal of Med Microbiol*, 296: p. 125-31.

Mascher et al., 2006 – Mascher T, Helmann J D and Unden G. (2006). Stimulus perception in bacterial signal- transducing histidine kinases. *Journal of Microbiol Mol Biol Rev*, 70: p. 910-38.

Marcie et al., 2005 – *Marcie B, Clarke MB and Sperandio V.* (2005). Transcriptional autoregulation by quorum sensing Escherichia coli regulators B and C (QseBC) in *enterohaemorrhagic E. coli* (EHEC) *Mol Microbiol,* 58(2), 441–455.

Messer et al., 2002 – *Messer W, Blaesing F, Majka J, Nardmann J, Schaper S, Schmidt A, Seitz H, Speck C, Tüngler D, Wegrzyn G, Weigel C, Welzeck M and Zakrzewska-Czerwinska J.* (2002). Functional domains of DnaA proteins. *Biochimie*, 81(8-9): p. 819-25.

Morigen et al., 2003 – *Morigen, Løbner-Olesen A and Skarstad K.* (2003). Titration of the *Escherichia coli* DnaA protein to excess data sites causes destabilization of replication forks, delayed replication initiation and delayed cell division. Mol Microbiol, 50: p. 349-362.

Morigen et al., 2005 – *Morigen, Molina F and Skarstad K.* (2005). Deletion of the datA site does not affect once-per-cell-cycle timing but induces rifampin –resistant replication. *Journal of Bacteriol,* 187: p. 3913-3920.

Sharma et al., 2010 – *Sharma V. K, Bearson S. M and Bearson B. L.* (2010). Evaluation of the effects of sdiA, a luxR homologue, on adherence and motility of *Escherichia coli* O157: H7. *Journal of Microbiol*, 156: p. 1303-1312.

Skarstad et al., 1986 – *Skarstad K, Boye E, Steen HB* (1986). Timing of initiation of chromosome replication in individual Escherichia coli cells. *EMBO J*, 5: 1711.

Boye, Løbner-Olesen, 1991 – *Boye E, Løbner-Olesen A* (1991). Bacterial growth control studied by flow cytometry. *Res Microbiol*, 142: 131–135.

Sperandio et al., 2003 – *Sperandio V, Torres A, Jarvis B, Nataro J and Kaper J.* (2003). Bacteria-host communication: the language of hormones. *Proc. Natl. Acad. Sci.* U S A, 100: p. 8951-8956.

Sperandio et al., 2002 – *Sperandio V, Torres A and Kaper J.* (2002). Quorum sensing *Escherichia coli* regulators B and C (QseBC): a novel two-component regulatory system involved in the regulation of flagella and motility by quorum sensing in *E. coli. Journal of Mol. Microbiol*, 43: p. 809-821.

Sperandio et al., 2001 – *Sperandio V, Alfredo G. Torres, Jorge A. Girgorn and Kaper J.* (2001). Quorum Sensing Is a Global Regulatory Mechanism in *Enterohemorrhagic Escherichia coli* O157:H7. *Journal of Bacteriol,* p. 5187–5197.

Sperandio et al., 1999 – *Sperandio V, Mellies J, Nguyen W, Shin S and Kaper J.* (1999). Quorum sensing controls expression of the type III secretion gene transcription and protein secretion in *enterohemorrhagic* and *enteropathogenic Escherichia coli. Proc. Natl. Acad Sci USA*, 96: p. 15196–15201.

Tomizawa, Selzer, 1979 – *Tomizawa J. and Selzer G.* (1979). Initiation of DNA synthesis in *Escherichia coli. Annu Rev Biochem*, 48: p. 999-1034.

Wold et al., 1994 – Wold S, Skarstad K, Steen HB, Stokke T, Boye E (1994). The initiation mass for DNA replication in *Escherichia coli* K-12 is dependent on growth rate. *EMBO J*, 13: 2097–2102.

Zyskind, Smith, 1986 – Zyskind J and Smith D. (1986). The bacterial origin of replication, *oriC. Cell.* 46(4): p. 489-90.

Published in the Slovak Republic
European Journal of Molecular Biotechnology
Has been issued since 2013.
ISSN: 2310-6255
E-ISSN: 2409-1332
2017, 5(2): 74-79

DOI: 10.13187/ejmb.2017.2.74
www.ejournal8.com

Phytochemical Analysis and Antibacterial Assay of Stem Bark of Anogeissus Leiogarpus

I.A.A. Ibrahim [a, *], M. M. Mohammad [a], A. A. Faisal [a], H. Musa [b], G. Altine [c], A. Bununu [c]

[a] Department of Science Laboratory Technology, School of Science and Technology, Abubakar Tatari Ali Polytechnic Bauchi, Nigeria
[b] Department of Microbiology, School of Science, Bauchi State University, Gadau, Bauchi, Nigeria
[c] Department of Agricultural Technology, College of Agriculture Bauchi, Nigeria

Abstract

Anogeissus leiocarpus is extensively used in *Nupe* traditional and folklore medicine to cure various human ailments. The preliminary phytochemical screening of the stem bark revealed the presence of flavonoids, soponins, steroids, terpeniods, cardiac glycosides and anthraquinones. In vitro antibacterial studies of the methanol and water extracts of the plant part were carried out on medically important bacterial strains including, Lacbacillus spp, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Bacillus spp, and Salmonella typhii using agar plug techniques. The results compared well with standard antibiotics (streptomycin) with susceptibly increasing with concentration. The extracts of the plant showed antibacterial activity, justifying their continued used in treatment of bacterial infections.

Keywords: *Anogeissus leiocarpus,* Lacbacillus spp, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Bacillus spp, Salmonella typhii.

1. Introduction

Anogeissus leiocarpus belongs to the family combretaceae (common name: Axle wood tree) is popular known as *marke* in Hausa northern Nigeria. Plant-drive compounds are a major area of interest to source for safer and more effected antibacterial agents (Longanga et al., 2000; Blench, Dendo, 2007; Musa et al., 2011).

A *leiocarpus* is used medically for the treatment of ascaricide, gonorrhea, general body pains, blood clots, asthma, coughing and tuberculosis (Nalule, Mbaria, Kimenju, 2013; Diab, Guru, Bhushan, Saxena, 2015). Information obtained from the Yurubas and south and eastern people of Nigeria illustrates that the plant is also used as an antimicrobial agent against bacterial infections (Blench, Dendo, 2007; Musa et al., 2011).

The leaves of the plant are used in Nigeria for the treatment of skin diseases and the itch of psoriasis. The powdered bark is applied to wounds, sores, boils, cysts and diabetic ulcers with good results. The powdered bark has also been mixed with 'green clay' and applied as an unusual face mask for serious blackheads (Longanga et al., 2000; Patil, Gaikwad, 2011; Olufunmilayo, 2017).

* Corresponding author
E-mail addresses: iliyasuibrahim@gmail.com (I.A.A. Ibrahim)

A *leiocarpus* is traditionally acclaimed to be effective in treating infections wounds in man and Animals (Akinpelu, 1999; Barku et al., 2013).

A *leiocarpus* in folk medicine is used for the treatment of skin infections, wounds. mouth infections, parasitic infections such as malaria, as well as jaundice (Nalule et al., 2013; Rajeshwar et al., 2016). Plant medicine was commonly used for traditional treatment of some infections disease to show significant antimicrobial activity (Ibisi et al., 2017; Eltayeb et al., 2018). Higher plant procedure hundreds to thousands of diver's chemical compound with different biological activities. Antibacterial active principles isolated from higher plants used in traditional medicine were developed in to drugs. A substantial number of drugs currently being used are discovered as a result of chemical studies directed of the isolation of the active substance from plants used in rational medicine (Eltayeb et al., 2016). This plant based system continue to play an essential role in health care, and it has been estimated by the world health organization that approximately 80% of the world's inhabitants rely mainly on traditional medicine for their primary health care (Diab et al., 2015).

Nigeria flora has over seven thousand, three hundred and forty and forty nine species of higher plants that had make serious impact on health and wealth of Nigerians and could be an enormous sources of foreign exchange for country (Musa et al., 2011).

Materials and methods

Preparation of reagents

Wagner's reagent

About 3.0g of potassium iodide was weighed and dissolved in about 40cm^{3}of distilled water. To the resulting solution of potassium iodide, 2.0g of iodide crystal was added and properly stirred to homogenize into solution. This was transferred into 100cm^3 volumetric flask and filled up to the mark with distilled water.

Potassium hydroxide

About 8.0g of potassium iodide was weighed and dissolved in about 600cm^{3}of dissolved water them transferred in to 100cm^{3}volumetric flask. This was filled to the mark with distilled water.

Lead acetate

10g of lead acetate was accurately weighted and dissolved in about 40cm^{3}of distilled water, this then transferred in to 100cm^{3}volumetric flask and filled to the mark with distilled water.

Sample collection and preparation

Fresh and healthy plant part of ***A. Leicarpus*** were collected from Rafin-Tambari, behind Abubakar Tatari Ali Polytechnic Bauchi, Bauchi State, Nigeria and authenticated by an agronomist in the same facility. The plant was washed, cut into small pieces and completely dried at room temperature (27^o) for two weeks. The dried plant materials were ground in to powder and stored in air tight glass bottles at room temperature prior to experiment.

Extraction

100g of the pulverize stem bark of dried plant part was macerated in 100ml of 70% methanol for 72hours with string. The extract was filtered through whatman No. 1 filter paper to remove all unextractable matter, including cellular materials and other constituents that are insoluble in the extraction solvent. The extracts were filtered through a whatman No 1. Filter paper and the filtrate concentrated to dryness using evaporator under reduced pressure.

Phytochemical analysis

Phytochemical analysis of the extract was conducted on the stem bark of A *leiocarpus* using standard methods. The presence of sugars, proteins, alkaloids, flavonoids, saponins, tannins cardiac glycosides, terpenoids and lipids were tested for.

Test for flavonoids

The presence of flavonoids was estimated using standard methods.

Test for tannins

2ml extract was added to 1% lead acetate a yellowish precipitate indicate the presence of tannins.

Test for Saponins

5ml extract was mixed with 20ml of distilled water then agitate in graduated cylinder for 15 minutes. Formation of foams indicates the saponins.

Test for cardiac Glycosides

Killer-killani test: plant extract treated with 2ml glacial acetic acid containing a drop of fetch. A brown colour ring indicated the presence of positive test.

Test for steroids

2ml ofscetic anhydride was added to 0.5mg of the extract of 2ml of $H_2 50_4$ The colour changed from boiled to blue or green indicating the presence of steroids.

Test for anthraquinones

0.5g extract was shaken with benzene (2.0cm^3) and filtered where necessary. 10% ammonia solution (4c m^3) was then added to the filtrate. The resulting mixture was shaken and the presence of a pink colour in the ammonia solution phase [lower layer] indicates the presence of Anthraquinones.

Bio – Assay

The extract was tested for antimicrobial activity using standardized agar dics diffusion methods. The bacteria were inoculated on the fresh media of nutrient agar slants incubated at 37^{o}C for 24 hours and were referred to as seeded broth. 0.3ml potion of the new culture was especially transferred in to Petri dishes containing 1m base medium, gently agitated and poured as over lay on assay plate containing 15m base medium.

The preparation was left to dry under hood, Different concentrations' of the extract were introduced to spots and streptomycin (200, 300, 400 mg/ml) was used as standard and a control experiment was set up by using drop of sterile water in place of different of concentrations extract.

The plates containing the bacteria, various concentrations of extract and the antibiotic used as standard as well as the control plates were allowed to stand for an hour at room temperature to allow the growth of the organisms to commence.

The plates were observed for zones of inhibition, which was measured in (mm), after 24 hours incubation at 3% in triplicate determination.

Extraction preparation for bio-assay

Antimicrobial activity of the aqueous and organic extract of the plant sample was evaluated by paper disc diffusion method of determination of antimicrobial activity, bacterial culture were subjected to 0.5ml for turbidity standard and inoculated on to nutrient agar plate (15cm diameter).

Preparation of media

28g of nutrient agar powder was suspended in 1 liter of distilled water, and boiled to dissolve completely and dispense as required. Sixty four (64g) of sabouraud dextrose agar (SNA) was suspended in 1 liter of distilled water and swilled continuously for even distribution, this was then sterilized in an autoclave at 12^{o}C for 15 minutes and allowed to cool.

Inoculation and application of extract

For the determination of antimycotic activity all the fungal isolations and Candida albicans were first adjusted to the concentration 0.9% normal saline and the spore of the other filamentous dextrose agar plate. Bacterial culture and the Candida albicans, were then incubated at room temperature (30-32^{o}C) for 48 hours. Paper disc impregnated with 20 ul of a solution of 10 mg/ml of ciprofloxacin and cotrimoxazole (for bacteria) and nystatin and amphotericin B for fungi as standards.

Minimum Inhibition Concentration (MIC)

The minimum inhibitory concentration (MIC) of the plant extract that showed inhibition in the antimicrobial screening was determined. The MIC was carried out by preparing the dried plant extract in different concentrations 10^{-2} mg/ml, 10^{3} mg/ml, and 10^{-6} mg/ml, respectively.

The different concentrations of the diluted extracts were filled inside the wells on the inoculated nutrient agar plats and allowed to stand for I hour for proper diffusion of the extract and them inoculated after which the lowest concentration that showed inhibition was checked for.

Result and discussion

Results

Table 1. phytochemical screening result of *A. leicarpus*.

S/N	Test of extract	Methanol extract	Water extract
1.	Flavonoids	+	+
2.	Tannins	+	+
3.	Sponins	+	+
4.	Steroids	+	+
5.	Terpenoids	+	+
6.	CardialGlyvosides	+	+
7.	Antthaquinones	-	-

Keys:-
+ = Present
- = Absent

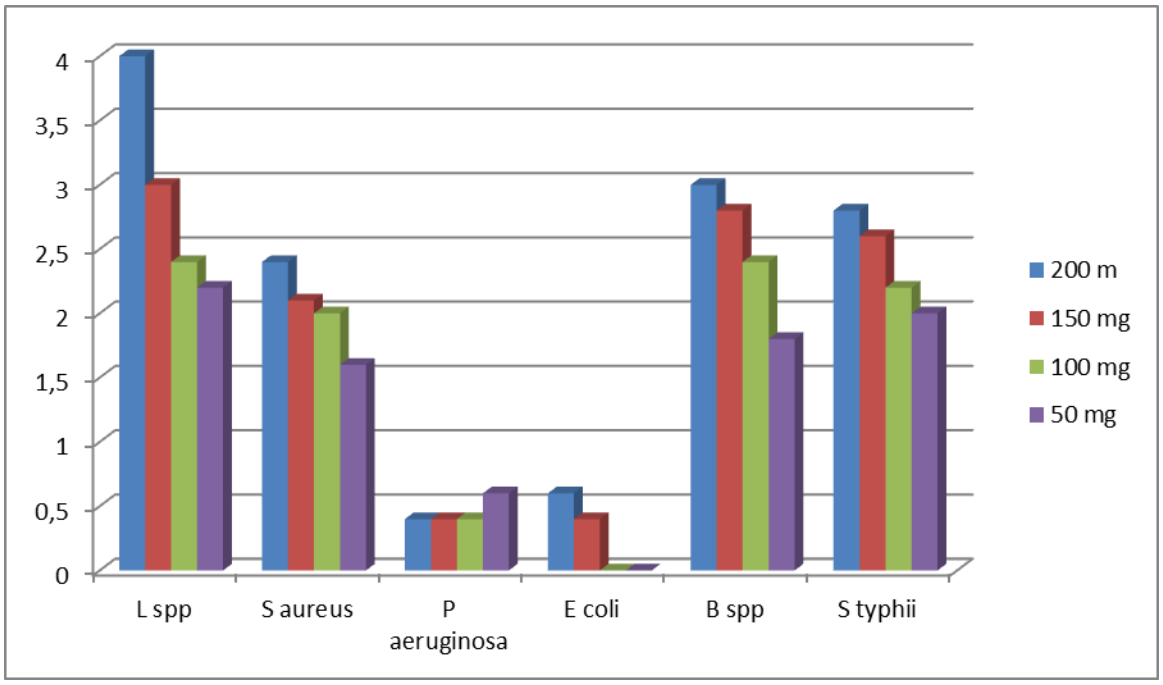


Fig. 1. Zone of inhibition [mm] of extract plant part against the test organism

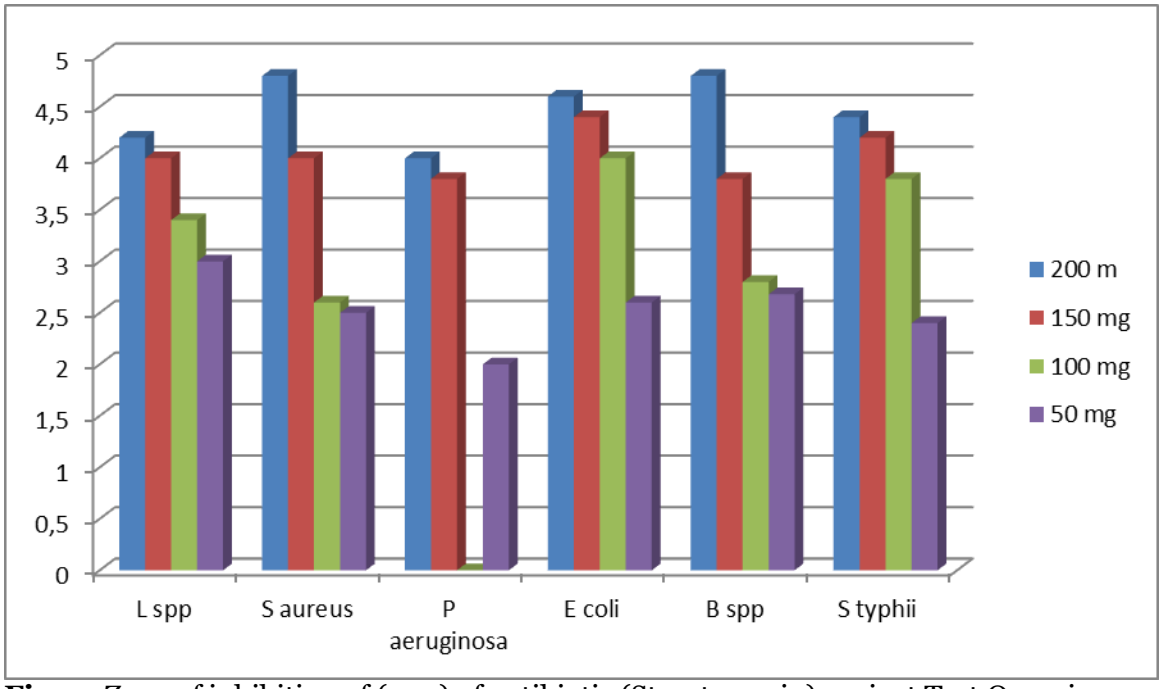


Fig. 2. Zone of inhibition of (mm) of antibiotic (Streptomycin) against Test Organism.

Discussion

The preliminary phytochemical analysis of the extracts revealed the presence of flavonoids. tannis, saponins, cardiac glycosides, terpenoids and anthraquinones as presented in the table 1 and fig. 1 and 2.

Susceptibility test of the methanolic extract of the tested plant part was positive on all tested organisms; comparable observations were made in similar work. It has been found that among all the tested organisms, the bacterial strain, lactobacilillus spp was found to be more susceptible to the plant extract by showing inhabitation as presented in table 2 and fig. 1 and 2.

The present study has shown that susceptibility increase with concentration as seen in both the extract and the standard. The extract compares favorably with the standard. The gradual increase in effect of extracts on test organisms with alteration of concentration has been reported by others (Ghamba et al., 2014; Bekele, 2015; Eltayeb et al., 2018).

References

Akinpelu, 1999 – *Akinpelu, D. A.* (1999). Antimicrobial activity of Vernonia amygdalina leaves. *Fitoterapia*, *70*(4), 432–434. https://doi.org/10.1016/S0367-326X(99)00061-1

Barku et al., 2013 – *Barku, V. Y. A., Opoku-Boahen, Y., Owusu-Ansah, E., Dayie, N. T. K. D., & Mensah, F. E.* (2013). In-Vitro Assessment of Antioxidant and Antimicrobial Activities of Methanol Extracts of Six Wound Healing Medicinal Plants. *Journal of Natural Sciences Research*, *3*(1), 74–80.

Bekele, 2015 – *Bekele, T.* (2015). Invivo Anti-Malarial Evaluation of Leaf Extract of Vernonia amygdalina Del. (Asteraceae) against Plasmodium berghei A Thesis submitted to Addis Ababa University, School of Graduate Studies, and Department of Biochemistry in Partial fulfillment of the.

Blench, Dendo, 2007 – *Blench, R., & Dendo, M.* (2007). Hausa names for plants and trees, (0), 8.

Diab et al., 2015 – Diab, K. A. E., Guru, S. K., Bhushan, S., & Saxena, A. K. (2015). In vitro anticancer activities of Anogeissus latifolia, Terminalia bellerica, Acacia catechu and Moringa oleiferna Indian plants. *Asian Pacific Journal of Cancer Prevention*, *16*(15), 6423–6428. https://doi.org/10.7314/APJCP.2015.16.15.6423

Eltayeb et al., 2016 – *Eltayeb, I. M., Abdel, H., Ali, R., & Muddather, A. K.* (2016). Madurella mycetomatis Susceptibility to Anogeissus leiocarpus Stem Bark Extracts. *American Journal of Research Communication*, *4*(3), 68–86.

Eltayeb et al., 2017 – *Eltayeb, I. M., Abdel, H., Ali, R., & Muddather, A. K.* (2017).

Antioxidant Activity and Cytotoxic Studies of Anogeissus leiocarpous Root, Leaf and Stem. *American Journal of Research Communication*, *4*(3), 52–67.

Ghamba et al., 2014 – *Ghamba, P. E., Balla, H., Goje, L. J., Halidu, A., & Dauda, M. D.* (2014). In vitro antimicrobial activities of Vernonia amygdalina on selected clinical isolates. *Int. J. Curr. Microbiol. App. Sci*, *3*(4), 1103–1113.

Ibisi et al., 2017 – *Ibisi, N. E., Okoroafor, D. O., & Amuta, C. C.* (2017). Comparative Study of Mild Steel Corrosion Inhibition of Piper Guineense Leaves Extract and Vernonia Amygdalina Leaves Extract in Concentrated Corrosive Medium. *IOSR Journal of Applied Chemistry*, *10*(5), 70–78. https://doi.org/10.9790/5736-1005027078

Longanga et al., 2000 – *Longanga, O., Vercruysse, A., & Foriers, A.* (2000). Contribution to the ethnobotanical, phytochemical and pharmacological studies of traditionally used medicinal plant in the treatment of dysentery and diarrhea in Lomela area, Democratic Republic of Congo (DRC). *J Ethnopharmacol*, *71*(3), 411–423.

Musa et al., 2011 – *Musa, M. S., Abdelrasool, F. E., Elsheikh, E. A., Ahmed, L. A. M. N., Mahmoud, A. L. E., & Yagi, S. M.* (2011). Ethnobotanical study of medicinal plants in the Blue Nile State, South-eastern Sudan. *Journal of Medicinal Plants Research*, *5*(17), 4287–4297.

Nalule et al., 2013 – *Nalule, A. S., Mbaria, J. M., & Kimenju, J. W.* (2013). In vitro anthelmintic potential of Vernonia amygdalina and Secamone africana on gastrointestinal nematodes. *Agriculture and Biology Journal of North America*, *4*(1), 54–66. https://doi.org/10.5251/abjna.2013.4.1.54.66

Olufunmilayo, 2017 – *Olufunmilayo, L. A.* (2017). Acute toxicity and hypoglycemic properties of ethanolic root extract of vernonia amygdalina. *International Journal of Current Research*, *9*(5), 50132–50138.

Patil, Gaikwad, 2011 – *Patil, U. H., & Gaikwad, D. K.* (2011). Ethno-pharmacological Review of a Herbal Drug : Anogeissus latifolia. *International Journal of Pharma Sciences and Research*, *2*(1), 41–43.

Rajeshwar et al., 2016 – *Rajeshwar, Y., Sreekanth, T., Kahsay, G., Hussen, D. A., & Eticha, T.* (2016). Hepatoprotective activity of Anogeissus latifolia bark in ethanol intoxicated rats. *The American Journal ofScience and Medical Research*, *1*(2), 198–201. https://doi.org/10.17812/ajsmr1210.Published

Published in the Slovak Republic
European Journal of Molecular Biotechnology
Has been issued since 2013.
ISSN: 2310-6255
E-ISSN: 2409-1332
2017, 5(2): 80-88

DOI: 10.13187/ejmb.2017.2.80
www.ejournal8.com

Autopoiesis Concepts for Chemical Origins of Life and Synthetic Biology

Stenogram of the popular lecture on the foreign bibliographic seminar

Oleg V. Gradov [a, *]

[a] Institute of Energy Problems of Chemical Physics RAS, Russian Federation

Abstract

The monograph (*Luisi P.L. "The Emergence of Life: From Chemical Origins to Synthetic Biology", 2010, Cambridge University Press, Cambridge, New York etc., 315 p.*) is a well-written, informative book providing a novel view on the interrelation between the abiogenesis as the natural origin of life and synthetic biology as the artificial synthesis of life. This concept is specially known as autopoiesis. As its name implies, it is a correlate of self-organization, but this word has quite a broad meaning in the literature. Consequently, some further restriction is required for this term in abiogenetic, as well as in "biogenetic" applications. There is, in fact, one basic reason for considering the abiogenetic problem in terms of self-organization theory. It follows from the extremely boundless complexity of biological systems.

Keywords: autopoiesis, autopoesis, chemical physics, synthetic biology, emergence of life, chemical origins of life.

1. Introduction

The monograph (*Luisi P.L. "The Emergence of Life: From Chemical Origins to Synthetic Biology", 2010, Cambridge University Press, Cambridge, New York etc., 315 p.*) is a well-written, informative book providing a novel view on the interrelation between the abiogenesis as the natural origin of life and synthetic biology as the artificial synthesis of life. This concept is specially known as autopoiesis. As its name implies, it is a correlate of self-organization, but this word has quite a broad meaning in the literature. Consequently, some further restriction is required for this term in abiogenetic, as well as in "biogenetic" applications. There is, in fact, one basic reason for considering the abiogenetic problem in terms of self-organization theory. It follows from the extremely boundless complexity of biological systems. The behavior of elementary biopolymers in cells is not less complicated, but it is well-known that, statistically, physicochemical steps leading to cooperative molecular network state are very complicated and stochastically improbable processes, requiring dozen billions years to achieve an appropriate adaptive coordination of molecular conformations and spatiotemporal synchronization of reaction pathways. Meanwhile, it is worth mentioning that the Earth age is 4.54 billion years (only!) on the 20-billion-year timeline of the Universe at best such. All this seems to convenience that life is almost impossible and,

* Corresponding author
E-mail addresses: gradov@chph.ras.ru (O.V. Gradov)

consequently, one may conclude that we do not exist at all. This is quite amusing, because the aforementioned strict result of positivistic scientific calculations, based on the rigorous statistical logic, leads us to absolutely undisguised solipsistic, subjective-idealistic and even immaterialistic conclusions! "This gives a contradiction, and the proof is complete" in terms of the predicate logic. It is hopeless to try to find a correct solution of the incorrect problem using an incomplete variance set. Another method uses various unverifiable and hardly relevant factors including hyperphysical and supernatural agents, such as eternal life transfer in multiple interconnected Universes and "directed panspermia" as a doctrine about the Earth life having been seeded by other civilizations. The above concept is almost identical with theological constructions about the intelligent design of life, based on dogmatic creationism, because this speculation way is also a satisfactory modus for the subjective overcoming of the time constraint problem in the ontology of life and its unexamined complexity.

The following simple reasoning may give us some insights into this question: there is a vitally important distinction between catalyzed and uncatalyzed processes. If we introduce a necessarily existing catalyst or an accelerant into the natural medium (e.g. the oxidation/reduction processes via an intermediary compound, known as energy carrier, or a physical factor by which the reaction rate constant may be shifted), we can avoid the inevitability of the statistically supernatural agents for "apologetics" of self-sustaining high organized life existence. There is enough experimental evidence for the fact that model "abiogenetic" processes take place in laboratory only under high-energy conditions or on the active surfaces of some mineral catalysts. That is plausible because abiogenesis is not the pre-Pasteurian "spontaneous generation" and, obviously, requires a special inducer for initiation and "energizing" of the following metabolic processes.

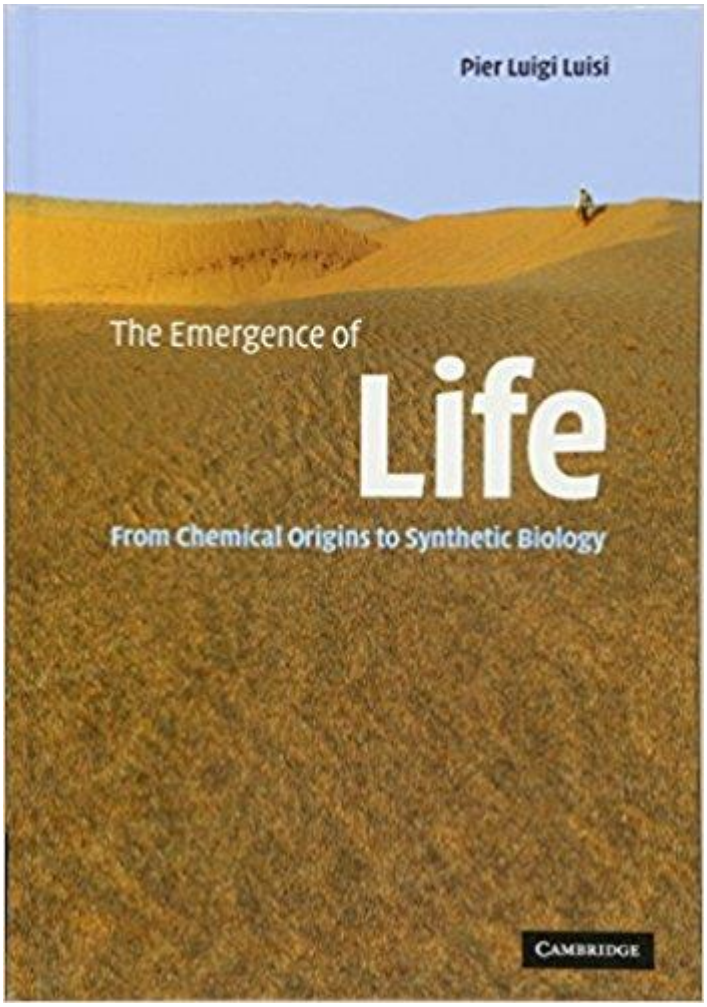


Fig. 1. 1-st edition (Luisi, 2010): Luisi P.L. "The Emergence of Life: From Chemical Origins to Synthetic Biology", 2010, Cambridge University Press, Cambridge, New York etc., 315 p. [ISBN-10: 0521821177 ISBN-13: 978-0521821179]

Most of our positions about abiogenesis come from experiments and, positively, unless there are experimental reasons for the contrary, it should be assumed that such processes simply do not occur without indispensable physical conditions. It seems reasonable to assume that those physical conditions may be interpreted as general inducers of abiogenesis. All such factors could produce unexpected effects on the abiogenetic process and they were also likely to take place within the ancient Earth or in cosmic conditions. The medium response to the effect depends on its sensitivity to the physical spectrum range used (that is known as spectral selectivity) or/and on selective recognition based on supramolecular chemistry principles, par excellence, complementary interactions as a form of early chemical specificity or selectivity of prebiological sensitive matter. Formally, the variety of such systems is endless, but we can see that most of laboratory abiogenesis simulations rely on and are based on the Earth conditions and the Earth organic life reconstruction. This limitation is of particular significance for synthetic biology and molecular biomimetics, because one is tempted to surmise that the Earth life is similar to all forms of life in the Universe and, consequently, search and simulation of the Earth life is based on the principles of the Earth life identification ("identical life of identical twins"). In fact, practically we do not really need any information about other possible forms of life, but it is not equivalent to its isomorphism to the organic life and to the statement, that other forms simply do not exist. In the before-mentioned widespread approaches to the abiogenesis modeling we shall meet another generalization of the same basic idea, because anyone can see, that this approach involves copying of substrate-dependent outward phenomena, but not the identification and imitation of the functional principles of biological processes. Indeed, it may not even be possible to imitate general life principles without general life function inducers! This is very strange, because the statements about impossibility of physical dynamics without an impulse and energy should look familiar to anyone who has studied physics in a high school, but the equivalent statement about biophysical dynamics initiation and, consequently, the need for an inducer of model dynamics in "artificial life" modeling and creation is not quite evident and generally accepted.

It is of great historical interest to trace the origins of this ridiculous situation. On the one hand, we can state that many authors make no difference between conservative and non-conservative self-organization mechanisms, but on the other hand, we can see that it is widely believed that forced induction of life processes is similar to "vis vitalis" or "entelechy" in archaic natural philosophy. This practice leads to a serious philosophical confusion, because similar scientists make the anti-idealistic, antivitalistic assumptions to avoid some undue associations, but these assumptions lead to mechanistic-idealistic conclusions, because they neglect a number of objective forces and factors without which any mechanism of abiogenesis becomes even more statistically incredible than it really is in practice. But this is not the way how it naturally happened, because specified inducers or "energizers" of the biological processes were not supernatural factors. Thus, it is unreasonable to expect any process to start without the objective inducers, but we also have no a priori reasons to expect that those processes were unearthly ones. Furthermore, it is therefore important to consider and understand the processes involved in geochemical and geophysical, astrochemical and astrophysical evolution before the general activation mechanism of the abiogenesis will completely emerge in our mind. But how can we understand this result without direct experiments on "environmental" (e.g. early Earth and cosmic conditions) reconstruction? And how good will be the simulation of the abiogenetic process without a full variance of this processes at the model scale that is incommensurable with the natural conditions? How should we then interpret the different scaling of the processes with unequal sets of probability samplings between the inducer-accelerated abiogenetic process and the inducer-free one? In order to keep our readers from misunderstanding, the following paragraphs outline the main methodological difficulties arising from modeling of the abiogenetic processes on our planet, but the arguments provided below can be equally applied to extraterrestrial forms of life, if any exist.

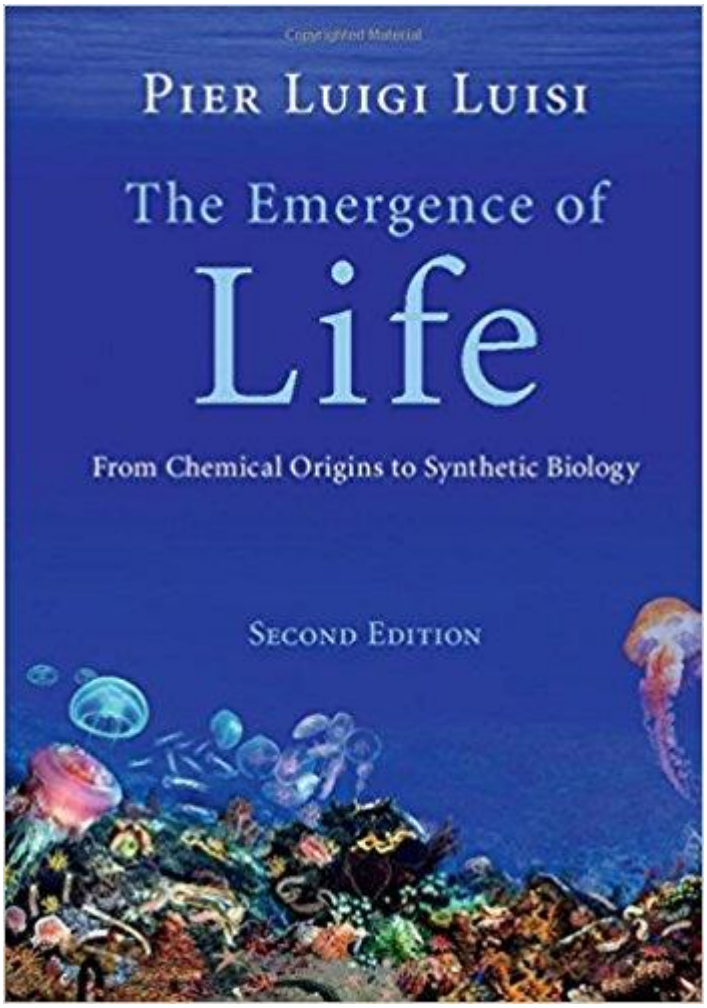


Fig. 2. 2-nd edition (Luisi, 2016) Luisi P.L. "The Emergence of Life: From Chemical Origins to Synthetic Biology", 2016, Cambridge University Press, Cambridge, New York etc., 478 p. [ISBN-10: 1107092396; ISBN-13: 978-1107092396]

The first methodological difficulty emerges as soon as we attempt to create artificial life as a model of the native product which is a result of a multimillion-year chemical evolutionary process, because there is no experimental foundation for extrapolation of "expressly-accelerated" abiogenetic principles on the natural evolution. The converse, though less trivial, is also true, as well as the fact that the forced aging of the photographic or polymeric material is not similar to the native aging of this material.

Nevertheless, the difference between the process rates does not exclude the equivalence of the process phenomenology at the atomic and molecular scale. This equivalence is based on the equivalence of physical mechanisms and laws for process accelerating in physicochemical kinetics. The scheme we will use is identical to that used for model reactor optimization: either we raise the temperature, or increase the particle collision rate, or even catalyze the process using chemical agents, the total result in the form of the reaction rate acceleration is expected to be the same. Yet it is not quite true to argue that those manipulations are fully equivalent - conversely, it is clear that the reaction pathways for different cases can be dissimilar. But that is exactly a qualitative difference between biological and non-biological process acceleration and, hence, a general discrepancy between biological and non-biological self-organization. In other words, the real difficulty lies in our insufficient knowledge about physical conditions of the early Earth or planetary reaction conditions, which determine a decision point for alternative selection of the accelerating way; and the main experimental imperfection arises from the statistical scale inconsistency of any artificial simulation tools with respect to the large-scale natural mechanisms of abiogenesis. This is the fundamental physical feature of any kind of geophysical, astrophysical

and other similar trends of local laboratory modeling, because geophysical method applies thermobarogeochemical (or seismochemical) approach to the process acceleration and astrophysical simulation method is substantially based on particle swarms and Monte-Carlo statistical approaches - i.e. physical tools for acceleration of chemical evolutionary processes, including those ones at the protobiological state. Probably, the most known technique is that involving plasma physics, e.g. Urey-Miller experiment, but it is only a method for plasma-chemical synthesis of organic compounds with high molecular weights. However, this method presupposes a detailed knowledge of the early Earth atmosphere chemistry and the search of sugars, amino acids and other abundant bioorganic chemical constituents of life. However, this selective methodology neglects a number of concomitant chemical processes resulting in the synthesis of alternative compounds which are not characteristic of modern bioorganic chemistry. A serious disadvantage of this method is the impossibility to accidentally synthesize and analyze a series of alternative compounds and to define them irrespectively as new life indicators, based on the strictly experimental "abiogenetic" conditions. In other words, the above way of interpreting possesses no predictive value! Strictly speaking, however, such a decision is meaningless because there are several ways of providing the interpretation of accelerated life emergence, but all of them are not falsifiable (or refutable) and all of them do not satisfy the scientific objectivity status. This avoids the problem of the need to decide whether abiogenesis is possible or not in this particular model case. To date, a full comprehensive and self-consistent theory of abiogenesis does not exist due to the lack of completely satisfactory models of life; but the absence of such a completely satisfactory theoretical model of life results from the ignoring of the basic mechanisms of abiogenesis. In other world, "vicious circle is virtuous circle".

Fig. 3. Prof. Dr. Pier Luigi Luisi

A pedantic experimentalist might wonder whether the only method available to us so far is a total impact on the experimental medium leading to numerous substances with the subsequent digging there in search of single minor compounds, whereas much research has been concentrated on the search of specific marker biochemical molecules in a set of artifacts and various molecular-chemical noise. Serious complications in many laboratory measurements usually arise from the choice of the marker compound. It is obviously impossible to gain a comprehensive knowledge

about the abiogenetic marker compound nature without a fundamental knowledge of the natural conditions during abiogenesis, since the bifurcation tree of molecular evolution can certainly follow a different path. This is not as simple as it may seem, since we can conclude, that the life chemistry character is a direct result of the geochemistry (or the "planetochemistry") of its abiogenetic conditions. Let us suppose, for example, that the Earth atmosphere was less reduced than one would expect at first, and thus the catalyzing clay mineralogenesis was distorted and shifted, the atmosphere chemistry was not good enough for the effective lighting propagation and the streamers of stratospheric discharges did not reach the near-earth area. In this case large-scale chemical processes of molecular evolution in this area would not resemble our life genesis background preconditions and precursors. And now let us consider a hypothetical experiment in which we can reconstruct the prehistoric world parameters mentioned here. We will then need a special argument to show that "another world is another life". The second experiment differs from the first one in several important ways. The first thing we can do is to change the composition of the atmosphere, and secondly we can substitute the material template for molecular precipitation and concentration including porous mineral intrusion for affine separation of the synthesized molecules based on the natural pseudo-chromatographic principles that are known to occur frequently under geochemical conditions. By a suitable choice of the solid phase of the mineral templates and the gas phase of the early atmosphere substituent it is possible to change the final "prebiotic" products (as it is known for industrial reactor design and optimization). Possibly, there is an important difference between "life as it is" and "life as it could be" and, consequently, between the abiogenesis modeling and the "strong Artificial Life" trend in "wet artificial life" modeling. In order to prove this result using combinatorial chemistry principles we should first calculate the probabilistic parameter for the desired product prevalence and also the statistical distribution of the other molecular structures. In many cases this will become obvious from physicochemical, e.g. spectral, investigation of our experimental result material. We turn our attention next to proving that spatiotemporal physicochemical kinetics and structural supramolecular properties of some active compounds similar to their biochemical prototypes also give evidence for replication and molecular evolutionary selection, but no prior knowledge of the chemical perquisites and the initial parameters is required. This experiment may seem artificial, but it clearly illustrates that the basic interpretation of the Urey-Miller experiments is opened to some criticism and the various results of this experiments can be appropriately interpreted in other hands. We would like to look at this result here from a slightly different point of view and, clearly, we aim to search for the better experimental principles and interpretation approaches to this controversial issue.

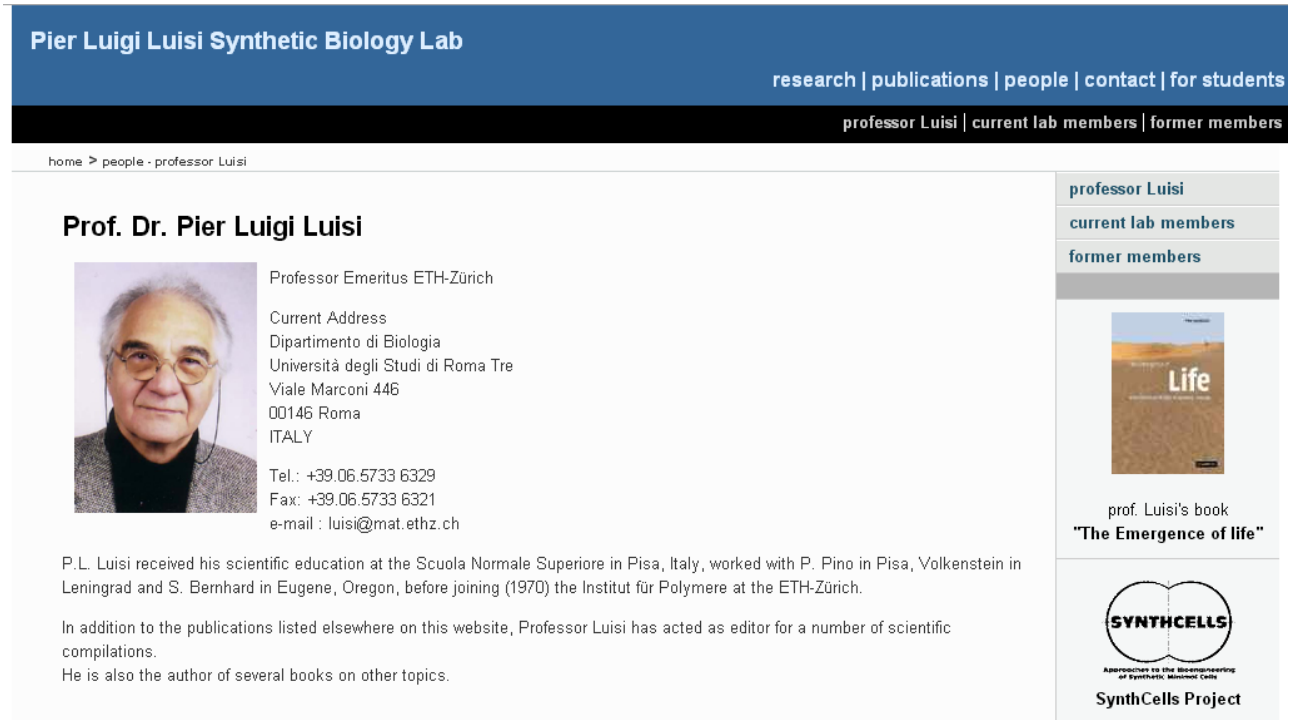


Fig. 4. Web-page of the Pier Luigi Luisi Synthetic Biology Lab (http://www.plluisi.org/luisi.html)

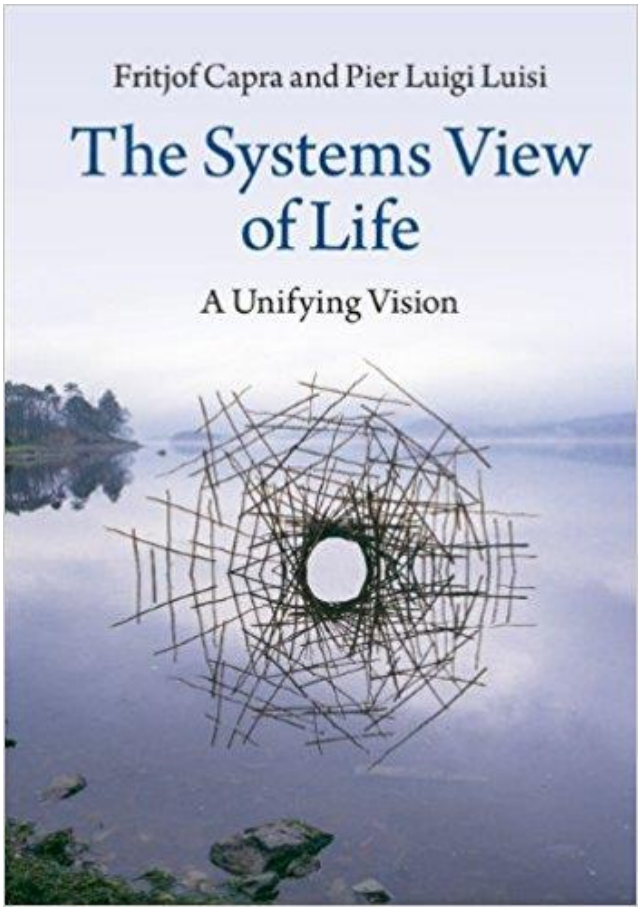


Fig. 5. "The Systems View of Life". Novel book in frame of the autopietic concepts

The question under consideration is also extremely fundamental for the emergence of biological organization principles if we consider the mechanisms more carefully. The classical Urey-Miller experiment discussed above is a special case of the generalized principle of the molecular life elements generation. This is why we were also able to obtain other "elements", but all such components can effect the origin of life only if they are sterically compatible, i.e. capable of providing chemical protocell organization. Nevertheless, the problem of prebiological supramolecular system complexation (which is directly interdeducible from the first "bichemical networking" problem) has not yet been solved satisfactorily. Here we present a necessary and sufficient condition for spatiotemporal complexation origin of biochemical structure dynamics and system reaction pathways. This will provide us the required characterization of cellular life unit formation principle which has not yet been discussed in the well-known standard variations of the abiogenetic experiments, since classical scholars considered only the proposed possible pathways, but did not examine the possibility of existing of the spatiotemporal reaction pathways which make them systematic. This is in fact a more complicated problem than the usual determination of molecular indicators of life in the "primordial soup" models, but the remarkable fact is that these necessary conditions are also sufficient for life! If we consider the real cellular life as a structural-heterogeneous multiphase dynamic system, we can see that the model life is impossible without compartmentalization and also without a "mover" of interfacial dynamics that should be synchronized in space and time. For this case, the only way is to synchronize its spatiotemporal dynamics in cooperative activity on the first stage of its mutual coexistence. From this analysis we should expect that the abiogenesis inducer should be the iducer not only of the simple chemical synthesis, but also of the metabolism initiation, interfacial compartmentalization and energy supply for these endergonic processes, for its coupled self-sustaining spatiotemporal dynamics. This follows at once from our physical considerations.

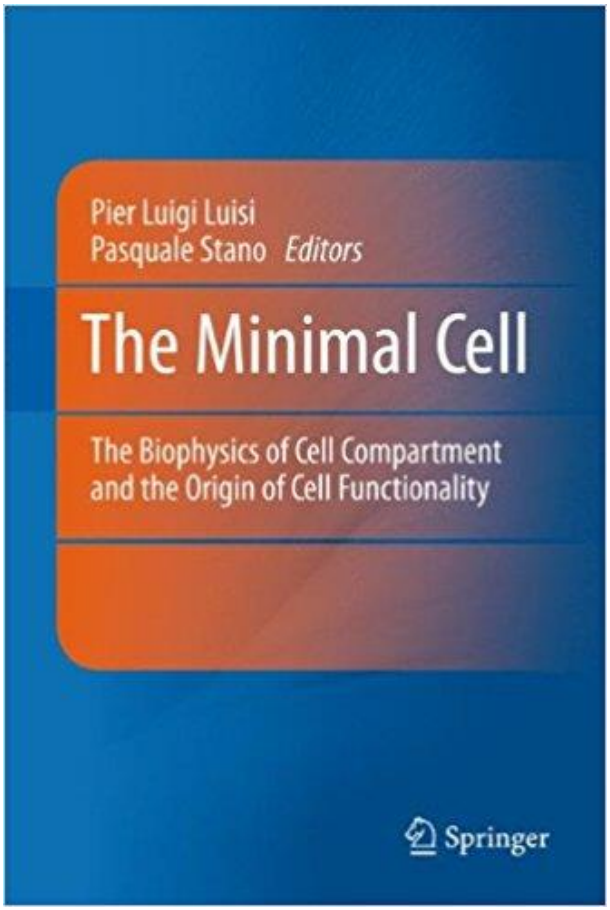


Fig. 6. The Minimal Cell: The Biophysics of Cell Compartment and the Origin of Cell Functionality, 2010 (Ed. by P.L. Luisi, P. Stano). One more book in frame autopoietic concept of abiogenesis, synthetic cell modeling and cellular morphogenesis (compartmentalization)

The "if" part is rather easy (no one objects if we say about the possibility, but not about the principal law), but the "only if" part is more difficult, because at the obsolete state of our knowledge, it is inevitable that in more complicated systems (than single atoms in classical quantum mechanics), it is necessary to take into account the existence of unexpected medium effects. Until the early 2000-s it was a common belief that we can synthesize protocell models using only structural (e.g. emulsification, inverse liposomal units, ultrasound processing) or only chemical (e.g. mineral templating or molecular imprinting, abiosynthesis or Urey-Miller experiments etc.) factors without their combining and complexation via energy sources. As a result, most of the workers in this field were about to consider abiogenesis as an equilibrium process and believed that metabolic reactions in protocell models could be initiated without any nonlinear dynamic mechanisms, i.e. only based on equilibrium chemical exchange principles. Well! But this practice leads to serious confusions, because the model system in this case tends to return to its thermodynamically stationary form; and it becomes tempting to assume that the abiogenetic process is actually an unrepeated, unknowable and wonderful (hyperphysical) phenomenon. However, it seemed inconceivable to the 19th-20th century biologists that such an evolutionary agnosticism is not quite good. The simplest way to overcome this difficulty is to conclude, that life is infinite in the Universe and might have had an extraterrestrial nature. The ideas involved in this speculations are so simple that seem not to require any proofs. This unfalsifiable idea is both self-contained and attractive, but its self-sufficiency is equal to the internal logic of the myth, because it is not thermodynamically deducible (since it appeals to transphysical infinity, that is not better, than spiritual eternity or immortality of life) as the statement about the possibility of the abiogenesis without an external inducer, which is also thermodynamically groundless (rejected by this methodological contrivance).

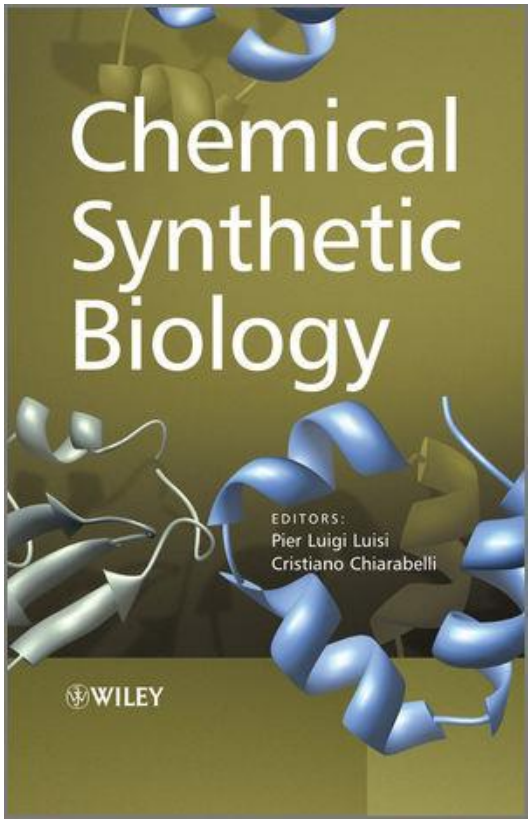


Fig. 7. "Chemical Synthetic Biology" (Ed. by P.L. Luisi, C. Chiarbeli). From the Back Cover (Chemical Synthetic Biology): "... chemical synthetic biology is concerned with the synthesis of new biological macromolecular structures (proteins and nucleic acids) and minimal life forms (semi-synthetic minimal cells) not found in nature, and - in contrast to other aspects of synthetic biology - without the use of genetic manipulation". (Chemical Synthetic Biology, 2011)

This becomes plausible from P.L. Luisi cosiderations, because his book, particularly, takes the reader through the transition from inanimate matter to life, i.e. from prebiotic chemistry to synthetic biology making the reader to believe into the concept named "autopoesis", which leads to the idea of compartments, discussed with an emphasis on vesicles and other highly orderly aggregates. Reading of his monograph suggests the difference between "models for cells" and "model of cells", because a true abiogenetic model should demonstrate the spontaneous increase in complexity from inanimate matter to the first cellular life forms, that makes possible the generation of the minimal cellular life within the laboratory.

References

Luisi, 2010 – *Luisi P.L.* (2010). The Emergence of Life: From Chemical Origins to Synthetic Biology, Cambridge University Press, Cambridge, New York etc., 315 p.

Luisi, 2016 – *Luisi P.L.* (2016). The Emergence of Life: From Chemical Origins to Synthetic Biology, Cambridge University Press, Cambridge, New York etc., 478 p.

Capra F. and Luisi P.L., 2014 – *Capra F. and Luisi P.L.* (2014). The systems view of life: A unifying vision. Cambridge University Press, 2014. [Kindle Edition]

The minimal cell: the biophysics of cell compartment and the origin of cell functionality, 2010 – Luisi, P. L., & Stano, P. (Eds.). (2010). The minimal cell: the biophysics of cell compartment and the origin of cell functionality. Springer Science & Business Media. [Kindle Edition]

Chemical synthetic biology, 2011 – *Luisi, P. L., Chiarabelli, C.* (Eds.). (2011). Chemical synthetic biology. John Wiley & Sons. [Kindle Edition]