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Original picture on front cover: Transylvanian smooth newt *in situ*

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Natural strategies of preventing anthracycline-induced cardiotoxicity – a review

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Abstract. Cardiotoxicity is one of the worst long-term effects of cancer therapy and doxorubicin is one of the main compounds responsible for cardiovascular complications. There are several biologically active compounds that can alleviate or prevent cardiotoxicity through multiple directions. Therefore, one of the aims of this paper is to emphasize the mechanisms by which several naturally occuring substances can improve the cardiac activity after drug-induced cardiotoxicity. The second part of this paper aims to update the molecular mechanism by which physical exercise and nutrition improve the life of a person with cardiovascular disease. The data collected showed that an active life and following a diet rich in polyphenols, coenzyme Q10, magnesium, may enhance the cardiovascular activity after exposure cu cardiotoxic drugs.

Keywords: cardiotoxicity, antioxidants, prevention, polyphenols

Introduction

Over the last decades, the number of cancer survivors has increased significantly due to progress in the methods of early detection and in antitumor treatment. For example, in the USA in 1975 the number of cancer survivors was 3.6 million, whereas in 2016 it was 15.5 million, and it is further estimated that in 2040 the number will be around 26.1 million (Shapiro, 2018). In Great Britain, the number of adult survivors has doubled in 40 years, and that of

children survivors has tripled (Cancer Research UK, 2014). Worldwide, the ten-year survival rate is approximately 50% for 20 of the most common cancers and over 80% for breast, uterus, lymphoma and melanoma cancers, and it is estimated that the rate will increase by 3% per year (Coleman *et al.*, 2011). In children, the survival rate exceeds 80% (Nathan *et al*., 2022).

Paradoxically, the survival of cancer patients is endangered by cardiotoxicity induced by antitumor treatment and especially by anthracyclines (doxorubicin, epirubicin, daunorubicin, idarubicin), which can be fatal. Doxorubicin (DOX) is highly efficient in treating solid and metastatic tumors (Sritharan and Sivalingam, 2021), malign lymphomas and leukemia (Benzer *et al.*, 2018). Cardiovascular complications of anthracyclines are due to high, cumulative doses, although problems may also occur at low doses and require discontinuation of treatment. Cardiotoxicity consists of the progressive decrease of the left ventricle ejection fraction (LVEF), which leads to the development of heart failure, high blood pressure, valvular disease, thromboembolism, arrhythmias (supraventricular and ventricular), myocarditis and pericarditis (Salvatici and Sandri, 2015; Nonaka *et al*., 2021).

Recently, several pharmacological compounds (modified anthracyclines, antioxidants, renin-angiotensin antagonists, cardioselective beta-blockers, statins etc.) have been tested for prophylaxis or treatment of cardiotoxicity, but their efficacy has not been proven by clinical trials (McGowan *et al.*, 2017). As such, natural compounds with cardioprotective properties have been studied and their result, both *in vitro* and *in vivo* animal studies, have shown that they could be used as an adjuvant treatment in cardiotoxicity.

The aims of this paper are 1) to emphasize the mechanisms of several naturally substances that can improve the cardiac activity after drug-induced cardiotoxicity and 2) to update the molecular mechanism by which physical exercise improves the life of a person with cardiovascular disease.

1. Natural alternatives for cardioprotection

Polyphenols are biologically active compounds found in plants, where they form defense systems, protecting them from pathogen invasions. There are thousands of polyphenols, which differ in the number of phenolic nuclei and hydroxyl groups, but also in the type of organic acids and carbohydrates attached. Polyphenols serve an important function in preventing cardiotoxicity mainly due to their antioxidant properties. One of the main mechanisms involved in the occurrence of cardiotoxicity is oxidative stress**,** therefore in order to mitigate its harmful effects polyphenolic treatments could be very effective (Wallace, 2003; Rana *et al*., 2022).

Resveratrol is a polyphenol found in the skin of grapes, berries and red wine. It has antioxidant, anti-inflammatory, anti-tumor properties and it is known as a modulator of lipid metabolism and inhibitor of LDL oxidation (Meng *et al.*, 2021). Resveratrol attenuates the effects of doxorubicin by reducing oxidative stress and lipid peroxidation, preventing apoptosis, and inhibiting cardiomyocyte autophagy without reducing the effectiveness of antitumor therapy (Hu *et al*., 2016; Tatlidede *et al*., 2009; Sin *et al*., 2015; Dutta *et al*., 2014).

Curcumin is the active compound extracted from the rhizomes of *Curcuma longa*, a plant popularly known as turmeric. It has many pharmacological and biological properties, such as antioxidant, anti-inflammatory, antitumor, antimicrobial and antifungal (Stanić, 2017). The cardioprotective effects of curcumin on doxorubicin treatment relies in improving cardiac biomarkers, reducing oxidative stress and inhibiting apoptosis (Imbaby et al., 2014).

Quercetin, which is found in all plant organisms except fungi and algae, is one of the most potent natural antioxidants (Hosseini, 2021). Its protective actions consist of chelating metal ions, removing free radicals, modulating lipid peroxidation and improving antioxidant systems, thus protecting DNA integrity (Muthukumaran *et al*., 2008; Matouk *et al*., 2013). Co-administered with doxorubicin, quercetin enhances the chemosensitivity of breast tumor cells to doxorubicin and in the liver potentiates the effects of doxorubicin in tumor cells, while protecting normal hepatocytes (Li *et al*., 2013; Wang *et al*., 2012). Quercetin attenuates cardiotoxicity by restoring the activity of antioxidant enzymes and lowering the concentration of proinflammatory cytokines, especially when co-administered with losartan (Matouk *et al*., 2013).

Epigallocatechin, the main polyphenol in green tea (*Camellia sinensis*), is known for its antioxidant and antitumor properties. Epigallocatechin improves both doxorubicin-induced and cisplatin-induced cardiotoxicity. After treatment with doxorubicin, it inhibits apoptosis and the formation of reactive oxygen species, both *in vitro* and *in vivo*. It prevents cardiotoxicity by inhibiting the formation of free radicals, apoptosis in normal cardiomyocytes and their overload with calcium (Shabalala *et al*., 2017). In the case of cisplatin treatment, epigallocatechin alleviates cardiotoxicity by improving histological parameters and the activities of antioxidant enzymes (Ibrahim *et al*., 2019).

Cardioprotective effects have also been noticed for other polyphenols, but they have a limited spread. For example, soybean genistein, parsley apigenin, celery and chamomile, rooibos aspalathin, hesperetin, naringenin and bergamot from citrus, oleuropein from olive leaves, extract of *Achillea fragrantissima* (Shabalala *et al*., 2017; Caressi *et al*., 2016; Hijazi *et al*., 2019).

To prevent or reduce anthracycline-induced cardiotoxicity, a number of plant extracts are being studied for their well-known cardioprotective effects. For example, extracts of *Citrus paradise*, *Passiflora incarnata*, *Angelica sinensis*,

Salvia miltiorrhiza, *Prunella vulgaris*, *Camellia sinensis*, *Phyllanthus urinaria*, *Acacia hydaspica* and *Centella asiatica* prevent cardiotoxicity through antioxidant properties. Extracts of *Astragalus polysaccharide*, *Azadirachta indica*, *Ganoderma atrum* and *Allium sativum* inhibit cardiomyocyte apoptosis and reduce DNA damage. Extracts of *Urtica parviflora*, *Flacourtia indica* and *Curcuma longa* decrease plasma triglycerides and LDL levels in doxorubicin-treated rats. Extracts of *Ginkgo biloba*, *Berberis vulgaris*, *Rhodiola rosea*, *Ixora coccinea*, *Vaccinium myrtillus* and *Panax notoginseng saponins* improve the contractile function of the left ventricle. Unfortunately, the plant extracts listed above will not soon be subject of clinical trials because 1) there is no information on the bioavailability of the active compounds in humans, and 2) most studies have been performed in tumor-free animals, so it is unknown whether these extracts affect antitumor activity of doxorubicin (Abushouk *et al*., 2017; Yu *et al*., 2018; Li *et al*., 2018; Xing *et al*., 2019; Afsar *et al*., 2019).

Coenzyme Q10, also known as ubiquinol, is synthesized in the human body in the smooth endoplasmic reticulum. It is involved in redox reactions in the mitochondrial respiratory chain, regulating the permeability of cell membranes, regulates endothelial function by regenerating vitamin E and preventing lipid peroxidation. Naturally, it is found in meat, fish, cereals, broccoli, spinach, and its synthetic form is available as capsules and solutions with different concentrations. Coenzyme Q10 is used to treat paracetamol poisoning, inflammatory diseases and heart failure. Recently, it has been shown to prevent doxorubicin-induced cardiotoxicity by reducing left atrium remodeling, improving myocardial oxygenation, reducing necrosis, fibrosis, and lipid infiltration into the heart muscle (Botelho *et al*., 2019).

Magnesium is involved in many intra- and extracellular processes. It has anti-inflammatory and antioxidant properties, inhibits apoptosis and reduces calcium overload in cardiomyocytes, and attenuates necrosis and apoptosis caused by CO poisoning. Food sources of magnesium are almonds, sesame seeds, bananas, cashews, and tofu. In doxorubicin-treated rats, magnesium reduces mortality, restores myocardial contractility, and improves antioxidant activity (Khalilzadeh *et al*., 2018).

Leucine is an essential branched chain aliphatic aminoacid used to attenuate proteolysis in hyperthyroidism, during treatment with dexamethasone and also in cancer. In experimental myocardial infarction, leucine serves as an alternative energy substrate, reducing arrhythmias and heart failure. Food sources of leucine are egg whites, meat, fish, dairy products, nuts, beans. Dietary supplementation with 5% leucine in doxorubicin-treated rats prevented the onset of cardiotoxicity by preserving the left ventricular ejection fraction and prevented fibrosis (Fidale *et al*., 2018).

2. Physical activity in the anthracycline-treated patients

In adults with cardiovascular disease, physical activity promotes recovery and improves the quality of life (Buckley *et al*., 2013). In cancer patients, physical activity during both treatment and recovery removes fatigue and depression and slightly improves the quality of life (Furmaniak *et al*., 2016; Lahart *et al*., 2018). For these reasons, the World Cancer Research Fund (WCRF) and the International Agency for Research on Cancer (IARC) recommend that cancer patients have at least 30 minutes of physical activity a day, at least five times a week (WCRF/AICR, 2007; Leitzmann *et al*., 2015). Physical activity may prevent or attenuate doxorubicin-induced cardiotoxicity, whether practiced before, during, or after cessation of treatment (Maia *et al*., 2017).

Although most of the information on cardioprotection provided by physical exercise originates from animal studies, it can be used as a starting point in clinical trials because it has been able to decipher, at least in part, the molecular mechanisms by which physical activity attenuates the side effects of doxorubicin. Physical activity decreases the excessive formation of reactive oxygen species induced by doxorubicin, by stimulating the activity of antioxidant enzymes such as glutathione peroxidase 1, catalase and superoxide dismutase. Aerobic exercise also stimulates the formation of heat shock proteins (HSPs) 60 and 72, which provide cardioprotection probably by controlling protein folding, preventing denaturation and aggregation of intracellular proteins, and accelerating the degradation of damaged proteins (Scott *et al*., 2011).

Physical activity inhibits doxorubicin-induced apoptosis by inhibiting Bax and caspase 3 activity and reducing p53 expression and apoptotic cardiomyocytes (Ascensão *et al*., 2005; Werner *et al*., 2008). Physical activity prevents doxorubicin-induced calcium overload of cardiomyocytes. Exercise restores myocardial contractile, systolic and diastolic function by stimulating calcium release from the smooth endoplasmic reticulum and modulating calpain activity (French *et al*., 2008).

Physical activity prevents doxorubicin-induced autophagy by inhibiting enzymes in its signaling pathway (Smuder *et al*., 2013). Also, it attenuates the accumulation of doxorubicin in the myocardium and thereby maintains its functions (Jensen *et al*., 2013).

There are a small number of studies in humans that have evaluated the ability of physical exercise to prevent or attenuate the cardiotoxicity of doxorubicin in women with early-stage breast cancer. The results are modest, as the studies included a small number of patients and the physical exercises were performed for short periods of time. A single exercise session before each dose did not affect the markers of cardiotoxicity (size and ejection

fraction of the left ventricle, troponin t and NT-proBNP), but improves hemodynamics, body weight and mood (Kirkham *et al*., 2018). Two sessions of aerobic and endurance exercise per week for two weeks before doxorubicin administration only improved plasma concentration of troponin and VO_2 (Howden *et al*., 2019).

Recently, several pilot studies have been proposed to evaluate the ability of exercise to prevent or treat anthracycline-induced cardiotoxicity. Lee *et al*. (2019) aimed to evaluate the effects of high-interval training on vascular function in breast cancer survivors undergoing anthracycline chemotherapy. Antunes *et al.* (2019) proposed a clinical study to determine whether a full-time exercise program (aerobic and endurance) accompanying doxorubicin treatment in 90 patients with invasive breast cancer attenuates cardiotoxicity. Foulkes *et al*. (2020) designed a study to determine if a 12-months structured exercise training can prevent cardiac morbidity in breast cancer patients. Diaz-Balboa *et al*. (2021) proposed a randomized trial to evaluate the impact of exercise-based cardiac prevention for the prevention of chemotherapy-induced cardiotoxicity in patients with breast cancer. No results of the proposed studies listed above have yet been published.

Conclusions

The development of cardiotoxicity complications brought by anticancer therapy is no longer a novelty. Although, finding solutions to reduce their harmful effects is still a challenge. The role of polyphenols and other biologically active compounds mentioned in the present paper, could significantly minimize the toxic effect of doxorubicin, by reducing producing of free radicals, improving myocardial oxygenation, reducing fibrosis, reducing calcium overload in cardiomyocytes and many more. As an additional cardioprotective strategy, performing physical exercise could accelerate the degradation of damaged proteins or stimulate the activity of antioxidant enzymes.

References

Abushouk A.I., Ismail A., Salem A.M.A., Afifi A.M., & Abdel-Daim M.M. (2017). Cardioprotective mechanisms of phytochemicals against doxorubicin-induced cardiotoxicity. *Biomed Pharmacother*, 90, 935-946.

Afsar T., Razak S., Almajwal A., Shabbir M., & Khan M.R. (2019). Evaluating the protective potency of Acacia hydaspica R. Parker on histological and biochemical changes induced by Cisplatin in the cardiac tissue of rats. *BMC Complement Altern Med*, 23, 19(1), 182.

Antunes P., Esteves D., Nunes C., Sampaio F., Ascensão A., & Vilela E. (2019). Impact of exercise training on cardiotoxicity and cardiac health outcomes in women with breast cancer anthracycline chemotherapy: a study protocol for a randomized controlled trial. *Trials*, 20(1), 433.

Ascensão A., Magalhães J., Soares J.M., Ferreira R., Neuparth M.J., & Marques F. (2005). Moderate endurance training prevents doxorubicin-induced in vivo mitochondriopathy and reduces the development of cardiac apoptosis. Am J *Physiol Heart Circ Physiol*, 289(2), H722-731.

Bacchetta, G., Farris, E., & Pontecorvo, C. (2012). A new method to set conservation priorities in biodiversity hotspots. *Plant Biosystems*, 146(3), 638-648.

Benzer F., Kandemir F.M., Ozkaraca M., Kucukler S., & Caglayan C. (2018). Curcumin ameliorates doxorubicin-induced cardiotoxicity by abrogation of inflammation, apoptosis, oxidative DNA damage, and protein oxidation in rats. *J Biochem Mol Toxicol*, 32(2).

Botelho A.F.M., Lempek M.R., Branco S.E.M.T., Nogueira M.M., de Almeida M.E., & Costa A.G. (2019). Coenzyme Q10 cardioprotective effects against doxorubicin-induced cardiotoxicity in Wistar rat. *Cardiovasc Toxicol*, [Epub ahead of print]

Buckley J.P., Furze G., Doherty P., Speck L., Connolly S., & Hinton S. (2013). BACPR stientific statement: British standards and core components for cardiovascular disease prevention and rehabilitation, *Heart*, 99(15), 1069-1071.

CancerResearchUK (2014). Cancer mortality in the UK 2012. In: Cancer Statistics Report. London UK.

Carresi C., Gliozzia M., Giancotta C., Scarcella A., Scarano F., & Bosco F. (2016). Studies on the protective role of Bergamot polyphenols in doxorubicin-induced cardiotoxicity. *Pharma Nutr*, 4(Suppl), S19-S26.

Ciocârlan, V. (2009) Flora ilustrată a României. Pteridophyta et Spermatophyta. *Ceres*, Bucureşti, 226 pp.

Coleman M.P., Forman D., Bryant H., Butler J., Rachet B., Maringe C, *et al*. (2011). Cancer survival in Australia, Canada, Denmark, Norway, Sweden, and the UK, 1995-2007 (the International Cancer Benchmarking Partnership): an analysis of population-based cancer registry data. *Lancet*, 377(9760), 127-138.

Diaz-Balboa E., Gonzalez-Salvado V., Rodriguez-Romero B., Martinez-Monzonis A., Pedreira-Perez M., Palacios-Ozores P., Lopez-Lopez R., Pena-Gil C., & Gozalez-Juanatey J.R. (2021). A randomized trial to evaluate the impact of exercise-based cardiac rehabilitation for the prevention of chemotherapy-induced cardiotoxicity in patients with breast cancer: ONCORE study protocol. *BMC Cardiovasc Disord*, 21(1), 165.

Dutta D., Xu J., Dirain M.L., & Leeuwenburgh C. (2014). Calorie restriction combined with resveratrol induces autophagy and protects 26-month-old rat hearts from doxorubicin-induced toxicity. *Free Radic Biol Med*, 74, 252-262.

Fidale T.M., Antunes H.K.M., Alex Dos Santos L., Rodrigues de Souza F., & Deconte S.R. (2018). Borges Rosa de Moura F. Increased dietary leucine reduces doxorubicin-associated cardiac dysfunction in rats. *Front Physiol*, 8, 1042.

Foulkes S.J., Howden E.J., Antill Y., Loi S., Salim A., Haykowsky M.J., Daly R.M., Fraser S.F., & La Gerche A. (2020). Exercise as a diagnostic and therapeutic tool for preventing cardiovascular morbidity in breast cancer patients-the Breast cancer Exercise InTervention (BREXIT) trial protocol. BMC Cancer, 20, 655-670.

French J.P., Hamilton K.L., Quindry J.C., Lee Y., Upchurch P.A., & Powers S.K. (2008). Exercise-induced protection against myocardial apoptosis and necrosis: MnSOD, calcium-handling proteins, and calpain. *FASEB* J, 22(8), 2862-2871.

Furmaniak A.C., Menig M., & Markes M.H. (2016). Exercise for women receveing adjuvant therapy for breast cancer. *Cochrane Database Syst Rev*, 9.

Gu J., Hu W., Song Z.P., Chen Y.G., Zhang D.D., & Wang C.Q. (2016). Resveratrol-induced autophagy promotes survival and attenuates doxorubicin-induced cardiotoxicity. *Int Immunopharmacol*, 32, 1-7.

Hijazi M.A., Jambi H.A., Aljehany B.M., & Althaiban M.A. (2019). Potential protective effect of Achillea fragrantissima against adriamycin-induced cardiotoxicity in rats via an antioxidant and anti-inflammatory pathway. *Biomed Res Int*, 5269074.

Hosseini A., Razavi B.M., Banach M., & Hosseinzadeh H. (2021). Quercetin and metabolic syndromeț: a review, *Phytother res*, 23(10), 5352-5364.

Howden E.J., Bigaran A., Beaudry R., Fraser S., Selig S., & Foulkes S. (2019). Exercise as a diagnostic and therapeutic tool for the prevention of cardiovascular dysfunction in breast cancer patients. *Eur J Prev Cardiol*, 26(3), 305-315.

Ibrahim M.A., Bakhaat G.A., Tammam H.G., Mohamed R.M., & El-Naggar S.A. (2019). Cardioprotective effect of green tea extract and vitamin E on Cisplatin-induced cardiotoxicity in mice: Toxicological, histological and immunohistochemical studies. *Biomed Pharmacother*, 113, 108731.

Imbaby S., Ewais M., Essawy S., & Farag N. (2014). Cardioprotective effects of curcumin and nebivolol against doxorubicin-induced cardiac toxicity in rats. *Hum Exp Toxicol,* 33(8), 800-813.

Jensen G.L., Compher C., Sullivan D.H., & Mullin G.E. (2013). Recognizing malnutrition in adults: definitions and characteristics, screening, assessment, and team approach. *JPEN J Parenter Enteral Nutr*, 37(6), 802-807.

Khalilzadeh M., Abdollahi A., Abdolahi F., Abdolghaffari A.H., Dehpour A.R., & Jazaeri F. (2018). Protective effects of magnesium sulfate against doxorubicin induced cardiotoxicity in rats. *Life Sci*, 207, 436-441.

Kirkham A.A., Eves N.D., Shave R.E., Bland K.A., Bovard J., & Gelmon K.A. (2018). The effect of an aerobic exercise bout 24 h prior to each doxorubicin treatment for breast cancer on markers of cardiotoxicity and treatment symptoms: a RCT. *Breast Cancer Res Treat*, 167(3), 719-729.

Lahart I.M., Metsios G.S., Nevill A.M., & Carmichael A.R. (2018). Physical activity for women with breast cancer after adjuvant therapy. *Cochrane Database Syst Rev*, 1.

Lee K., Kang I., Mack W.J., Mortimer J., Sattler F., Salem G., Lu J., & Dieli-Conwright C.M. (2018). Effects of high-intensity interval training on vascular endothelial function and vascular wall thickness in breast cancer patients receiving anthracycline-based chemotherapy: a randomized pilot study. *BMJ*, 177(2), 477-485.

Leitzmann M., Powers H., Anderson A.S., Scoccianti C., Berrino F., & Boutron-Ruault M.C. (2015). European Code against Cancer 4th Edition: Physical activity and cancer. *Cancer Epidemiol*, 39 Suppl 1, S46-55.

Li S.Z., Li K., Zhang J.H., & Dong Z. (2013). The effect of quercetin on doxorubicin cytotoxicity in human breast cancer cells. *Anticancer Agents Med Chem*, 13(2), 352-355.

Li W.J., Zhang X.Y., Wu R.T., Song Y.H., & Xie M.Y. (2018). Ganoderma atrum polysaccharide improves doxorubicin-induced cardiotoxicity in mice by regulation of apoptotic pathway in mitochondria. *Carbohydr Polym*, 202, 581-590.

Maia T.N., de Araujo G.B.R., Teixeira J.A.C., Junior E.D.A.J., & Dias K.P. (2017). Cardiotoxicity of doxorubicin treatment and physical activity: A systematic review. *Int J Cardiovasc Sci*, 30(1), 70-80.

Matouk A.I., Taye A., Heeba G.H., & El-Moselhy M.A. (2013). Quercetin augments the protective effect of losartan against chronic doxorubicin cardiotoxicity in rats. *Environ Toxicol Pharmacol*, 36(2), 443-450.

McGowan J.V., Chung R., Maulik A., Piotrowska I., Walker J.M., & Yellon D.M. (2017). Anthracycline chemotherapy and cardiotoxicity. *Cardiovasc Drugs Ther*, 31(1), 63-75.

Meng T., Xiao D., Muhammed A., Deng J., Chen L., & He J. (2021). Anti-inflammatory action and mechanisms of resveratrol *Molecules*, 26(1), 229.

Muthukumaran S., Sudheer A.R., Menon V.P., & Nalini N. (2008). Protective effect of quercetin on nicotine-induced prooxidant and antioxidant imbalance and DNA damage in Wistar rats. *Toxicology*, 243(1-2), 207-215.

Nathan P., Kulkarni K., & MacDonald T. (2022). Incidence and risk factors of anthracycline-induced cardiotoxicity in long-term survivors of pediatric cancer: A population-based cohort study. *Ped Hem Oncol J*, Journal pre-proof, DOI: https://doi.org/10.1016/j.phoj.2022.06.004.

Nonaka M., Hosoda H., & Uezono Y. (2021). Cancer treatment-related cardiovascular disease: Current status and future research priorities. *Biochem Pharmacol*, 190, 114599.

Rana A., Samtiya M., Dhewa T., Mishra V., & Aluko R.E. (2022). Health benefits of polyphenols: a concise review, *J Food Biochem*, e14264.

Salvatici M., & Sandri M.T. (2015). Identifying cancer patients at risk for cardiotoxicity. *Future Oncol*, 11(14), 2077-2091.

Scott J.M., Khakoo A., Mackey J.R., Haykowsky M.J., Douglas P.S., & Jones L.W. (2011). Modulation of anthracycline-induced cardiotoxicity by aerobic exercise in breast cancer: Current evidence and underlying mechanisms. *Circulation*, 124(5), 642-650.

Shabalala S., Muller C.J.F., Louw J., & Johnson R. (2017). Polyphenols, autophagy and doxorubicin-induced cardiotoxicity. *Life Sci*, 180, 160-170.

Shapiro C.L. (2018). Cancer survivorship. *N Engl J Med,* 379(25), 2438-2450.

Sin T.K., Tam B.T., Yung B.Y., Yip S.P., Chan L.W., & Wong C.S. (2015). Resveratrol protects against doxorubicin-induced cardiotoxicity in aged hearts through the SIRT1-USP7 axis. *J Physiol*, 593(8), 1887-1899.

Smuder A.J., Kavazis A.N., Min K., & Powers S.K. (2013). Doxorubicin-induced markers of myocardial autophagic signaling in sedentary and exercise trained animals. *J Appl Physiol*, 115(2), 176-185.

Sritharan S., & Sivalingham N. (2021). A comprehensive review on time-tested anticancer drug doxorubicin. *Life Sci*, 278, 119527.

Stanić Z. (2017). Curcumin, a compound from natural sources, a true Scientific challenge - A review. *Plant Foods Hum Nutr*, 72(1), 1-12.

Tatlidede E., Sehirli O., Velioğlu-Oğünc A., Cetinel S., Yeğen B.C., & Yarat A. (2009). Resveratrol treatment protects against doxorubicin-induced cardiotoxicity by alleviating oxidative damage. *Free Radic Res*, 43(3), 195-205.

Wallace K.B. (2003). Doxorubicin-induced cardiac mitochondrionopathy. *Pharmacol Toxicol*, 93(3), 105-115.

Wang G., Zhang J., Liu L., Sharma S., & Dong Q. (2012). Quercetin potentiates doxorubicin mediated antitumor effects against liver cancer through p53/Bcl-xl. *PLoS One*, 7(12):e51764.

WCRF/AICR. (2007). Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global Perspective, AICR, Washington, DC.

Werner C., Hanhoun M., Widmann T., Kazakov A., Semenov A., & Pöss J. (2008). Effects of physical exercise on myocardial telomere-regulating proteins, survival pathways, and apoptosis. *J Am Coll Cardiol*, 52(6), 470-482.

Xing J.J., Hou J.G., Liu Y., Zhang R.B., Jiang S., & Ren S. (2019). Supplementation of saponins from leaves of Panax quinquefolius mitigates cisplatin-evoked cardiotoxicity via inhibiting oxidative stress-associated inflammation and apoptosis in mice. *Antioxidants* (Basel), 8(9).

Yu J., Wang C., Kong Q., Wu X., Lu J.J., & Chen X. (2018). Recent progress in doxorubicin-induced cardiotoxicity and protective potential of natural products. *Phytomedicine*, 40, 125-139.

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A comparative assessment of artificial and natural energy drinks in the epididymal and testicular milieu

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Abstract. Artificial and natural energy drinks are both taken for increased energy, physical stamina, and alertness, although they differ in composition. This study investigated the effects of artificial and natural energy drinks on the testicular milieu in male pubertal rats.

Eighteen Wistar rats were randomly divided into 3 groups of 6 rats each and all animals had access to food *ad libitum*. Group 1: (control) received water only; Group 2: (artificial energy drink- AED) received AED; Group 3: (natural energy drink- NED) received NED. A dose of 1.41ml/day/150g animal was administered and this lasted for 28 days. Sperm and testicular variables, biochemical parameters, and hormonal assays were carried out.

There were significant decreases in the levels of testosterone, Lactate dehydrogenase, glucose, 3β-Hydroxysteroid dehydrogenase, and 17β- Hydroxysteroid dehydrogenase activities in AED and NED groups when compared to the control group. There was a marked increment in sperm abnormalities in the NED group when compared to AED and control groups. Also, the intake of AED led to an elevated level of Glucose-6-phosphate dehydrogenase compared to the control while a significant reduction was observed in the NED group when compared to the AED group. Artificial and natural energy drinks although consumed for strength and vigor distorted epididymis and testicular integrity via alteration of the testicular metabolism, lowering sperm quality and androgenic hormones in pubertal male Wistar rats.

Keywords: energy drink, jaggery, sperm quality, steroidogenic enzymes, lactate dehydrogenase.

Introduction

The emergence of energy or power drinks has been over three decades now, and they differ from tea, coffee, soft drinks, fruit drinks, and sports drinks. Energy drink as the name implies was invented to build up energy and strength, for alertness, and to improve mood. Studies reveal that these drinks are perhaps targeted at adolescents (Reissig *et al*., 2009), and there are other studies pinpointing the fact that males are more affiliated with energy drinks than females (Friis *et al*., 2014; Dillion *et al*., 2019).

The main ingredients in energy drinks include water, taurine, guarana, glucuronolactone, added sugars, and a high concentration of caffeine which is a nitrogenous compound of the alkaloid family which has concealed physiological effects such as stimulating the central nervous system (Campbell *et al*.,2013; Fields *et al*., 2015). Popular energy drinks could be referred to as artificial energy drinks because they contain added sugars as well as caffeine which varies between 50-505 mg per can or bottle (Keaver *et al*., 2017). These values appear extremely high when compared to a 250ml cup of coffee which contains 80-120mg of caffeine or a cup of tea which contains 60mg of caffeine. Although the safe limits of caffeine consumption remain to be determined, there are suggestions from data that the maximum recommended intake of caffeine per day in adolescents should be 100mg/day and in adults, it could be up to 400mg/day (Heckman *et al*., 2010). Apart from the added caffeine content of energy drinks, other components such as guarana contain caffeine which is not usually mentioned as part of the caffeine contents thus the caffeine content of such drinks might be higher than what is listed on the drink packs thereby causing caffeine toxicity (Duchan *et al*., 2010). There are other constituents added to artificial energy drinks claimed to boost energy and mental alertness and the relationships between these components and caffeine in these drinks are not known (De Sanctis *et al*., 2017).

There exist other types of energy drinks which boost the body's energy needs as well as supply nutrition to the body. These groups of energy drinks appear less popular but are from natural sources and do not contain added sugars or artificial caffeine. They could be referred to as natural energy drinks and examples include kvass and jaggery. Jaggery also referred to as *gur, panela, kokuto, hakura,* and *rapadura* is processed from sugarcane juice. It is a natural sweetener produced without the use of chemicals (Nath *et al*., 2015). It is made up of longer chains of sucrose. It produces heat and gives instant energy to the body (Rao and Singh, 2021). The energy it gives lasts for a long time, and it is harmless to the body (Kumar and Singh, 2019). Jaggery contains large amounts of iron during its preparation, and it, therefore, has anti-anemic properties. It also contains reducing sugars, minerals, vitamins, proteins, and

phenols apart from its sucrose content. Jaggery is one of the products of sugarcane obtained in unrefined form (Kumar and Singh, 2019) and this explains the medicinal and nutritional properties of jaggery brought about by the presence of phenolic compounds. The procedure by which jaggery is made is the term used by the Food and Agriculture Organization of the United Nations (FAO, 1994) to describe a traditional slightly processed sweetener derived from sugar cane.

In recent years, jaggery is being used globally as part of food processes and as an energy drink. The current generation of youths and adolescents appear to prefer energy drinks which keep them "on the go". Energy drinks continue to gain increasing acceptability, especially among male folks. This study was therefore designed to compare the effects of an artificial energy drink with a natural one (jaggery) on male reproductive functions in Wistar rats.

Materials and methods

Energy drinks

An energy drink (commercial name not disclosed) containing 31.5mg caffeine per 100ml, niacin (3.0mg), vitamin B_6 (0.3 mg), vitamin B_{12} (0.3µg) with an energy value of 283kj/67kcal was used for the study. Jaggery sugar (Golden Sugar Company Limited) was also used for the study.

Animals

Ethical approval was obtained from the College of Medicine, University of Lagos Animal Care and Use Research Ethics Committee (CMUL/ACUREC/ 07/21/876). Experimental procedures relating to the use of animals were by the EU Directive 2010/63/EU for animal study and the study conformed with the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guideline (2010). Eighteen (18) male Wistar rats (150-170g) were obtained from the Central Animal House, University of Lagos. They had access to rat feed and water and these animals were acclimatized for one week to laboratory conditions before the commencement of the administration of the drinks. The rats were kept in standard laboratory conditions of relative humidity, dark/light cycle, and temperature.

Experimental design

The control (Control) group received distilled water (vehicle) daily. The artificial energy drink (AED) group was administered a dose of 1.41ml/day/ 150g of animal body weight of the energy drink daily for 4 weeks through oral

gavage and the natural energy drink (NED) group was administered 1.4 ml/ 150g body weight of the jaggery daily for 4 weeks through oral gavage. The artificial energy drink was calculated based on the dose equivalent to one can of energy drink (250mL) by a human adolescent while jaggery solution (1.25 w/v) was prepared every day. Both energy drinks were diluted with water. The animals were not restricted to food consumption in any way. The body weight and food intake were measured weekly.

Blood collection and serum preparation

After the last day of administering the energy drinks, the rats were sacrificed using pentobarbital sodium anesthesia (50mg/kg, i.p). Blood samples were collected by cardiac puncture into plain sample bottles and the blood was allowed to stand for 30 min thereafter they were centrifuged at 3000rpm for 5min. The supernatant was decanted from the centrifuged blood and frozen at -20^{0}C. Right testis and seminal vesicle tissues were homogenized in phosphate buffer solution, centrifuged at 4,000rpm, and the supernatant separated and stored at -20^{0}C for analysis.

Biochemical assays

Testosterone, Luteinising Hormone (LH), and Follicle Stimulating Hormone (FSH) were assessed by enzyme-linked immunosorbent assay (ELISA) using ELISA assay kits (Acc-Bind Elisa microwell monoband (USA). Testicular cholesterol level was determined using spectrophotometric methods and laboratory kit reagents (Randox Laboratory Ltd, UK) were used for the analysis, and their absorbance was read using a UV-Vis spectrophotometer (DREL 3000 HACH) while testicular glycogen content was determined by harvesting and cleaning the testes of the animals. Afterward, known weights of testes were homogenized in ice-cold trichloroacetic acid (deproteinizing) solution and incubated for 15 minutes in a water bath. After discarding the precipitate, the supernatant was mixed with sulphuric acid and heated for 5 minutes and the absorbance was read with an ELISA reader (Biobase Bioindustry Co. Ltd., Shandong, China) at 620 nm wavelength. Standard glycogen (Sigma; St. Louis, MO, USA) was also prepared and employed for the standard curve. Glucose-6-phosphate was assayed with a Glucose-6-phosphate kit (Sigma; St Loius, MO, USA). Serum lactate dehydrogenase (LDH) and fructose were measured through enzyme colorimetric procedures with reagents purchased from Randox Laboratory Ltd (Antrim, UK). The catalytic property of LDH leading to reversible oxidation of L-lactate to pyruvate, mediated by the hydrogen acceptor, NAD+, is harnessed as a basis of the measurement of LDH activity.

The rate of production of NADH that changes the optical density of the sample was measured spectrophotometrically at 340 nm. The conversion of pyruvate to lactate or the reverse reaction of oxidation of L-lactate to pyruvate can be monitored spectrophotometrically. The analysis of fructose levels was as follows: 20 µl of seminal plasma was mixed thoroughly with 220 µl distilled water, and later deproteinized with 50 µl of ZnSO4 and 50 µl of NaOH. After 15 min of incubation, it was centrifuged at 2500 rpm and 200 µl of clear supernatant was mixed with Indole reagent followed by 32% hydrochloric acid. The mixture was incubated at 60°C for 20 min and after cooling, readings were taken at 470 nm (Karvonen and Malm, 1955). The terminal fasting blood glucose level of the rats was determined using Accu-Check Active (manufacturer: Roche Diagnostics, Pvt Ltd., Mumbai, Maharashtra, India).

Sperm analysis

The analysis of sperm function was carried out as described in a previous study (Adekunbi *et al*., 2016). Briefly, the caudal epididymis was harvested, and the epididymis was cut into pieces in 1 ml of 37^0C of normal saline solution. A drop of the solution was placed on a glass slide and covered with a coverslip and placed under a microscope at x40. The sperm motility was based upon; oscillatory or stationary, slow progression or rapidly progressive, vibrating movements, and these were expressed in percentages. The sperm morphology was determined as follows: a smear preparation was made (with formal saline) and was stained with 1 percent eosin stain, and it was allowed for 20 -30 minutes to allow staining to occur. A hundred (100) sperms per animal were morphologically examined at x100 magnification, the abnormal sperms were categorized based on the presence of irregular heads, detached tails, midpiece bending, and double tails. The sperm count was done using the enhanced Neubauer hemocytometer and was expressed as million/ml of suspension.

Statistical analysis

Data were presented as means ± SEM. Statistical analysis was carried out by analysis of variance (ANOVA) supported by the Newman-Keuls test when pair-wise comparison was done between the groups. The analysis was done using version 5.0 (GraphPad Software, San Diego California, USA). The level of statistical significance was placed at $p<0.05$.

Results

Effects of artificial and natural energy drinks on body weight and feed intake in male Wistar rats

All animals had increased body weights in their respective groups. There was however a significant reduction in the body weight of the NED group when compared to the control and AED groups respectively (Fig.1).

The food intake in the AED and NED groups was smaller ($P<0.05$) compared to the control group throughout the period of the experiment. The food intake in the natural energy drink (NED) group was also smaller when compared to the artificial energy drinks (AED) group.

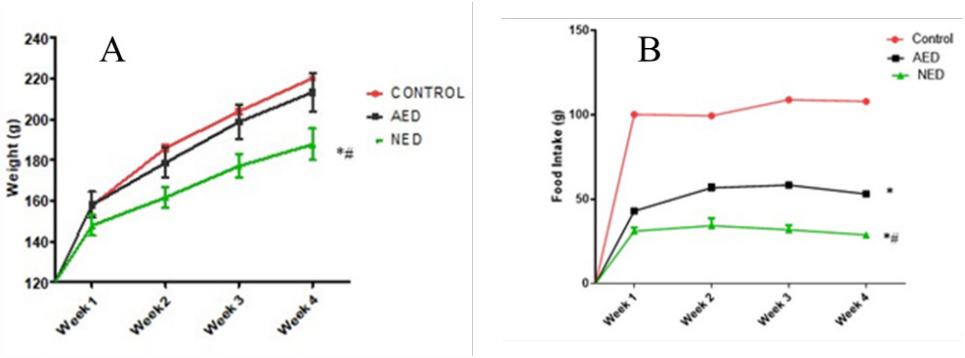

Fig.1. A shows reduced mean body weights of rats administered NED when compared to the control and AED groups. Values are expressed as mean ± SEM of 6 rats per group, *p <0.05 vs control, # p<0.05 vs AED. Natural Energy Drink (NED), Artificial Energy Drinks (AED); **B** shows reduced food intake of rats administered NED when compared to control and AED groups as well as when AED was compared to control. Values are expressed as mean ± SEM of 6 rats per group, *p <0.05 vs control, # p<0.05 vs AED. Natural Energy Drink (NED), Artificial Energy Drinks

Effects of artificial and natural energy drinks on fasting blood glucose level and seminal vesicle fructose in male Wistar rats

There was a significant decrease in the fasting blood glucose level in the AED and NED groups when compared to the control group. There was however no significant difference in the seminal vesicle fructose levels of the AED and NED groups when compared with the control (Fig. 2).

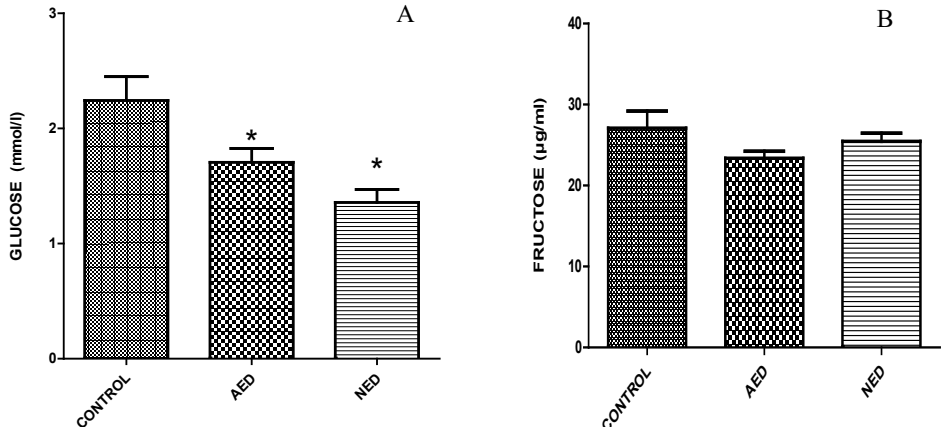

Fig 2. A shows reduced fasting blood glucose levels characterized rats in the AED and NED groups when compared to the control group, while **B** shows there were no changes in the seminal fructose levels. Values are expressed as mean ± SEM of 6 rats per group, *p <0.05 vs control. Natural Energy Drink (NED), Artificial Energy Drinks (AED).

Effects of artificial and natural energy drinks on 3-beta hydroxysteroid dehydrogenase (3β-HSD) and 17-beta hydroxysteroid dehydrogenase (17β-HSD) in male Wistar rats

A significant decrease in the 3-beta hydroxysteroid dehydrogenase (3β-HSD) activity was observed in the AED and NED groups when compared to the control group. Also, a significant decrease in the 17-beta hydroxysteroid dehydrogenase (17β-HSD) activity was observed in the AED and NED groups when compared to the control group. There was, however, a significant increase in the 17-beta hydroxysteroid dehydrogenase (17β-HSD) activity observed in the NED group when compared to the AED group (Fig. 3).

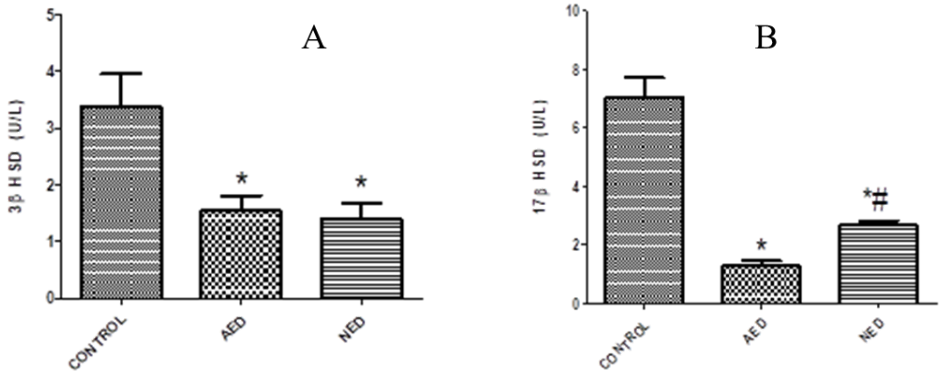

Fig 3. A, B show decreased 3β-HSD and 17β-HSD activities characterized rats in the AED and NED groups compared to the control group and **B**, also shows an increased 17β-HSD activity characterized rats in the NED group when compared to the AED group. Values are expressed as mean ± SEM of 6 rats per group, *p <0.05 vs control, # p<0.05 vs AED. Natural Energy Drink (NED), Artificial Energy Drinks (AED), 3-beta hydroxysteroid dehydrogenase (3β-HSD), 17-beta hydroxysteroid dehydrogenase (17β-HSD).

Effects of artificial and natural energy drinks on Lactate dehydrogenase (LDH) activity and Glucose-6-phosphate dehydrogenase (G6PD), testicular glycogen, and testicular cholesterol levels in male Wistar rats

A significant decrease in the Lactate dehydrogenase activity was seen in AED and NED groups when compared to the control group. There was a significant increase in the Glucose-6-phosphate dehydrogenase (G6PD) level in the AED group compared to the control group. Furthermore, there was a significant decrease in the Glucose-6-phosphate dehydrogenase level in the NED group compared to the AED group. There was no significant difference in the testicular glycogen level of the AED and NED groups when compared to the control. A significant decrease in the testicular cholesterol level was observed in the NED group when compared to the control group as well as in the NED group when compared to the AED group (Fig. 4).

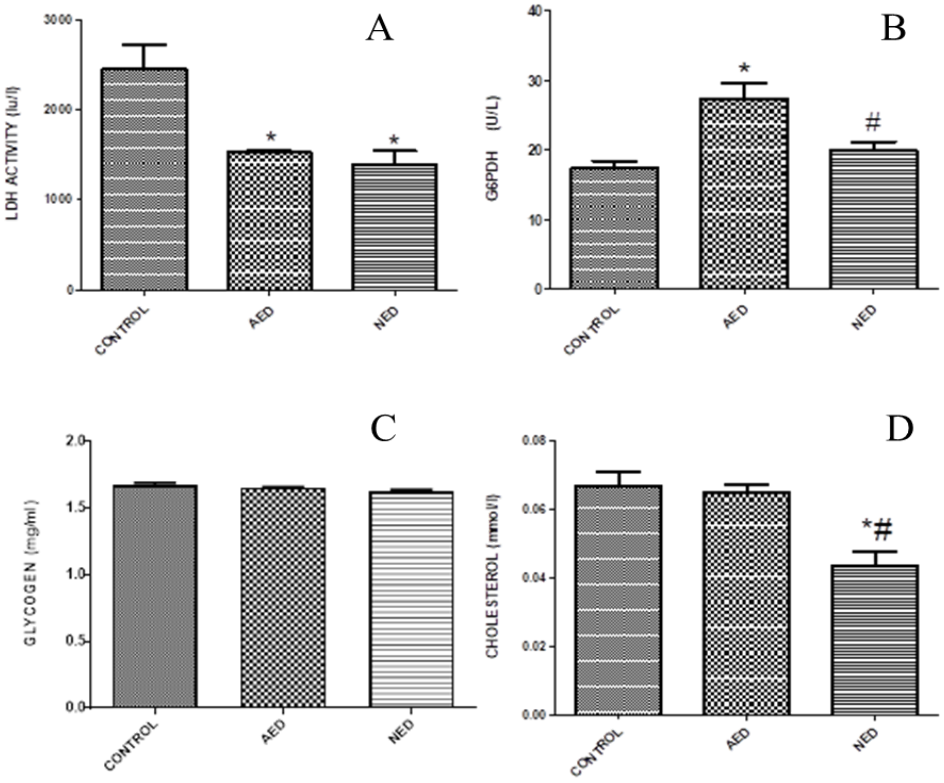

Fig 4. A shows reduced LDH activity characterized rats in AED and NED groups compared to the control group. **B** shows increased G6PD level was observed in animals in the AED group compared to the control group but a decrease in G6PD level was observed in animals in the NED group compared to the AED group. **C** shows no significant difference in the glycogen levels of rats in the AED and NED groups compared to the control group. **D** shows that reduced testicular cholesterol level was observed in the NED group when compared to the control group and also in the NED group when compared to the AED group. Values are expressed as mean ± SEM of 6 rats per group, *p <0.05 vs control, # p<0.05 vs AED. Natural Energy Drink (NED), Artificial Energy Drinks (AED), Lactate dehydrogenase (LDH), Glucose-6-phosphate dehydrogenase (G6PD).

Effects of artificial and natural energy drinks on serum hormonal profile in male Wistar rats

Administration of the artificial energy drink (AED) and the natural energy drink (NED) led to reduced testosterone levels in the AED and NED groups when compared to the control group. There was however a significant

increase in the testosterone level of the NED group when compared to the AED group. A significant decrease in the luteinizing hormone level was observed in the NED group when compared to the control and AED groups. There was however no significant difference in the follicle-stimulating hormone levels of the AED and NED groups when compared to the control (Fig. 5).

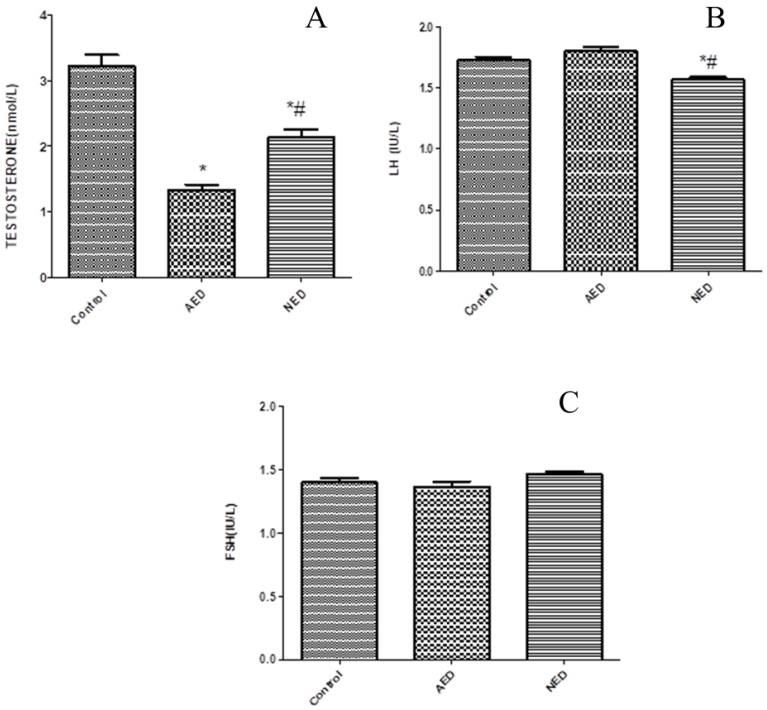

Fig 5. A shows reduced testosterone levels characterized rats in the AED and NED groups when compared to the control group but an increase in the testosterone level characterized rats in the NED group when compared to the AED group; **B** shows reduced LH level was observed in rats in the NED group when compared to the control and AED groups. **C**- shows there was no significant difference in the FSH levels. Values are expressed as mean ± SEM of 6 rats per group, *p <0.05 vs control, # p<0.05 vs AED. Natural Energy Drink (NED), Artificial Energy Drinks (AED), Luteinizing Hormone (LH), Follicle-Stimulating Hormone (FSH).

Effects of artificial and natural energy drinks on epididymal sperm characteristics in male Wistar rats

Sperm motility was significantly reduced in the NED group when compared to the control and AED groups while there was no significant

difference in the sperm count of the NED and AED groups when compared to the control group. The abnormal sperm morphology significantly increased in the NED group when compared to the control and AED groups (Tab.1).

Table 1. Effects of artificial and natural energy drinks on sperm variables in male Wistar rats**.**

	CONTROL	**AED**	**NED**
Sperm motility (%)	50.20 ±0.92	53.50 ±1.34	45.67±1.56 *#
Sperm count(million/ml)	63.78 ± 3.99	65.62 ± 3.30	61.20 ± 3.49
Abnormal sperm morphology (%)	13.33± 0.84	13.83± 0.98	21.17± 0.75*#

Values are expressed as mean ± SEM of 6 rats per group, *p <0.05 vs control, # p<0.05 vs AED. Natural Energy Drink (NED), Artificial Energy Drinks (AED).

Discussion

This study examined the impact of artificial and natural energy drinks on male reproductive functions. This becomes vital considering the growing concerns about male youngsters' preference for energy drinks. In this study, it appeared that high sucrose consumption is associated with the control of food intake as reported in another study (Adekunbi *et al.,* 2016). The artificial energy drink contains added sugars while the natural energy drink contains natural sugars. Sucrose satiating effects have been observed in previous studies (Anderson and Woodend, 2003; Tappy and Lē, 2009) and it was reported that sucrose satiating effect results in a high feeling of fullness as a result of the sweet taste of sugar (Lavin *et al.,* 2002). It is expected that food intake should match body weight. In this study, animals had increased body weights in their respective groups. There was however a significant reduction in the body weight of the NED group when compared to the control and AED groups respectively. There are conflicting data in the literature concerning sugar consumption on body weight gain in experimental animals; while some authors have reported an increased weight gain in rats, others have reported no significant changes or reductions (Adekunbi *et al.,* 2016; Driescher *et al.,* 2019). In this study, the significant reduction in body weight of the NED group compared to the control and AED groups appear to be a consequence of reduced food intake. Other components in these energy drinks could have roles to play regarding body weight and food intake regulation. The natural drink (jaggery) has been reported to assist in body weight control which could

be the reason for the reduction in body weight of the NED group when compared to the control group (Rao and Singh, 2021). Although the body weight of the AED group was not affected when compared to the control, there was a significant reduction in food intake in the group. Caffeine which is one of the important components of artificial energy drinks improves weight maintenance because of its thermogenic property. It also suppresses appetite and energy intake (Harpaz *et al.,* 2017; Correa *et al.,* 2018). Caffeine intake also decreases food intake in males (Graneri *et al.*, 2021).

There were no signs of systemic toxicity observed in the animals administered both natural and artificial energy drinks, evaluating sperm variables is however an essential step for predicting reproductive toxicity in males (Nallella *et al.,* 2006). According to this study, there was an increased percentage of aberrant sperm morphology in the NED group when compared to control and AED groups, motile sperms were also decreased in the NED group when compared to control and AED groups, while there was no significant difference in the sperm count among groups (Table 1). This result agrees with a study that suggested that a high intake of sucrose might lead to an increase in the percentage of abnormal sperm (Adekunbi *et al.,* 2016) and energy drinks contain high levels of sucrose as well (Jaffé, 2015). Abnormal sperms could also result from likely alterations during spermiation because it is released from the Sertoli cells into the lumen of the seminiferous tubules (Esteves, 2015).

The hypothalamic-pituitary-gonadal (HPG) axis is a standard negative feedback control. As blood testosterone level rises, the luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels should decrease. In this study, there was a significant decrease in the testosterone level of the AED and NED groups when compared to the control. The blood testosterone level of the NED group was significantly increased compared to AED. The LH level was significantly decreased in the NED group when compared to the control and AED groups. There was no significant difference in the FSH levels of the groups when compared to the control (Fig 5). The following inferences can be made from these results. Caffeine, a component of artificial energy drinks has been reported to reduce testosterone production (Al-Eryani *et al.,* 2018). The decrease in the testosterone level of the AED group was not affected by LH and FSH levels. This infers that the action of AED may not be through the gonadotropins but directly on the testes. There was an increase in the blood testosterone of the NED group when compared to the AED group which also resulted in the decrease of the blood LH levels in the NED group when compared to the control and AED groups respectively although there was no significant difference in the FSH level of the NED group. The testosterone level

of the NED group unlike the AED group appears to be stimulated perhaps partially by the gonadotropin-releasing hormone (GnRH) which resulted in reduced LH hormone secretion. Jaggery consumption might however be a better option for an energy drink than artificial energy drinks. Testosterone promotes spermatogenesis thus the reduction in testosterone level might be the result of the alterations reported in the sperm variables. The abysmal levels of the hormones may alter reproductive functions and may be a potential causative factor of male infertility consequently.

Lactate dehydrogenase is an indicator enzyme of carbohydrate metabolism needed for germ cell production and differentiation which will eventually result in active glycosylation by the Sertoli cells which secrete lactate as the main energy substrate for spermatids and spermatocytes (Akomolafe *et al.,* 2017). In this study, there was a decrease in LDH activity in AED and NED groups when compared to the control. The low LDH activity would inhibit androgen production (El-Kashoury e*t al.,* 2010) and low sperm action (Akomolafe *et al.,* 2017).

There was a significant increase in the testicular glucose-6-phosphate dehydrogenase (G6PD) level in the AED group when compared to the control. A study reported that an increase in glucose-6-phosphate dehydrogenase (G6PD) level would restore the redox balance in endothelial cells exposed to a high amount of glucose (Zhang *et al*., 2012),. According to this study, however, serum glucose was significantly reduced. The increase in G6PD level thus observed in this study was modulated in an entirely different pathway in the AED group and it was not a result of improved NADPH level which is required in the maintenance of tissue integrity. This scenario is different from the decreased G6PD level observed in the NED group when compared to the AED group. This reduced G6PD level would obstruct spermatogenesis and reduce androgen production (Anuja *et al.,* 2010).

Glycogen is the main source of energy for sperm cells, and it is directly proportional to the steroid hormone level (Govardhan and Changamma, 2014). In this study, there was no significant difference in the testicular glycogen levels in the AED and NED groups when compared to the control. Imbalance in glycogen and fructose contents in the testis and seminal vesicle could lead to germ cell apoptosis or degeneration as well as a decrease in the number of mature and motile spermatozoa (Kamal *et al.,* 1993; Kuramori *et al.,* 2009) as observed in the study.

In this study, the fasting blood glucose levels in both AED and NED groups significantly decreased when compared to the control group. A study also reported reduced fasting glucose after sugar-sweetened beverage consumption (Driescher *et al.,* 2019). Artificial and natural energy drinks have sugars as part of their constituents. Jaggery for instance also contains fructose

which has been reported not to affect blood glucose adversely when consumed in moderate amounts (Gaby, 2005). The animals in this study could probably be in a state of mild hyperglycemia which was why the effect of the glucose level was masked. This is because it has been reported that sucrose in drinking water as opposed to when taken in solid form may lead to adiposity in rodents (Kawasaki *et al.,* 2005). The cause of this however is not clear. It may however be because the level of sucrose, when delivered in water, is much higher than in the solid form (Togo *et al.,* 2019). When rodents consume sugar in drinking water, there is usually increased adiposity and impaired blood glucose homeostasis (Togo *et al.,* 2019). The significant decrease in the fasting glucose level in the AED group is however not in agreement with the study by (Bukhar *et al.,* 2012) that reported that consumption of artificial energy drinks led to a significant rise in serum glucose. The significant increase in the serum glucose levels in this study may be because of the duration of exposure to artificial energy drinks. This study exposed the animals to an artificial energy drink for 4 weeks, whereas in another study the animals were exposed to an artificial energy drink for 6 weeks (Bukhar *et al.,* 2012). The significant increase in the serum glucose levels in these studies may also be because of the age of the male rats used. In this study, pubertal rats were used, whereas, in another study, adult male rats were used (Bukhar *et al.,* 2012). In this study, there was also a significant decrease in the serum glucose level in the NED group compared to the control group. This finding is not in agreement with another study. The authors stated that the consumption of jaggery would lead to an increase in serum glucose (Patel *et al.,* 2011). The significant increase in the serum glucose levels in this study may be because of the duration of exposure to the natural energy drink. This present study exposed the animals to natural energy drinks for 4 weeks, whereas in another study, the animals were exposed to jaggery for 16 weeks (Patel *et al.,* 2011). The reason why the glucose levels in the AED and NED groups were significantly reduced when compared to the control group requires further investigation.

Cholesterol is a known precursor involved in steroidogenesis, a decrease in testicular cholesterol concentration would thus lead to a decreased production of testosterone and will impair fertility (Gatsing *et al.,* 2010). In this study, there was a significant decrease in the testicular cholesterol level in the NED group when compared to the control and AED groups (Fig. 4). The potassium that is present in jaggery is responsible for reducing cholesterol levels (Rao and Singh, 2021). The cholesterol level of the rats in the AED group was not affected because according to some authors the presence of taurine in artificial energy drinks prevents the decrease in testicular cholesterol levels (Chen *et al.,* 2016; Schuchowsky *et al.,* 2017).

In this study, there was a significant decrease in the testicular 3-beta hydroxysteroid dehydrogenase (3β-HSD) activity in both the AED and NED groups when compared to the control (Fig 3). This finding signifies that the consumption of both artificial and natural energy drinks caused a decrease in the activity of this important steroidogenic enzyme, which resulted in a decrease in testosterone synthesis in the Leydig cells of the testes and could thus impair fertility.

There was a significant decrease in the 17-beta hydroxysteroid dehydrogenase (17β-HSD) activity in the testicular homogenate of the AED group when compared to the control group (Fig. 3). This steroidogenic enzyme catalyzes the final step in the biosynthesis of the testosterone pathway (Rebourcet *et al.,* 2020). The finding from this study signifies that the consumption of artificial energy drinks will cause a decrease in the steroidogenesis of testosterone in the Leydig cells. There was a significant decrease in the 17-beta hydroxysteroid dehydrogenase (17β-HSD) activity in the NED group compared to the control group. This finding from the study signifies that the consumption of natural energy drinks could also cause a decrease in testosterone synthesis. In this study, there was a significant increase in the 17-beta hydroxysteroid dehydrogenase (17β-HSD) activity in the NED group compared to the AED group. This finding also shows that natural energy drink consumption could cause a significant increase in 17-beta hydroxysteroid dehydrogenase (17β-HSD) activity when compared to artificial energy drink consumption. Artificial energy drink consumption might cause a larger decline in testicular steroidogenesis of testosterone than natural energy drink consumption.

Conclusions

In conclusion, the results of this study establish that the consumption of both artificial and natural energy drinks would negatively affect testicular-metabolic characteristics, androgenic hormones, and sperm functions. The energy drinks disrupted steroidogenesis and the synthesis of testosterone and gonadotropins and altered sperm quality. The AED and NED thus have anti-gonadal properties.

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References

Adekunbi, D.A., Ogunsola, O.A., Oyelowo, O.T., Aluko, E.O, Popoola, A.A., & Akinboboye, O.O. (2016). Consumption of high sucrose and/or high salt diet alters sperm function in male Sprague–Dawley rats. *Egypt. J. Basic Appl. Sci,* 3 (2), 194-201. https://doi.org/10.1016/j.ejbas.2016.03.003.

Akomolafe, S.F., Oboh, G., Akindahunsi, A.A., & Afolayan, A.J. (2017). Ethanol-induced male infertility: Effects of aqueous leaf extract of Tetracarpidium conophorum. *Andrologia,* 00:e12759. https://doi.org/10.1111/and.12759.

Al-Eryani, F.S., Kelany, A.M., Amin, H.A., & Shazly, H.F. (2018). Histological and Physiological Studies on the Effects of Some Energy Drinks on Male Rats. *Int. J. Pharm. Res. Allied Sci,*7(1),165-176.

Anderson, H., & Woodend, G. (2003). Consumption of sugar and the regulation of short-term satiety and food intake. *Am. J. Clin. Nutr,* 73, 843S-849S.

Anuja, M.N., Nithya, R.N., Rajamanickam, C., & Madambath, I. (2010). Spermatotoxicity of a protein isolated from the root of Achyranthes aspera: a comparative study with gossypol. *Contraception,* 82(4),385-90. doi: 10.1016/j.contraception.2010.04.011.

Bukhar, H.M., ElSawy, N.A., & Header, E.A.(2012). Biological Effect of High Energy Drink on Normal and Hyperglycemic Rats. *Pak. J. Nutr,* 11, 301-309. doi: 10.3923/pjn.2012.301.309.

Campbell, B., Wilborn, C., La Bounty, P., Taylor, L., Nelson, M.T., Greenwood, M., Ziegenfuss, T.N., Lopez, H.L.,Hoffman, J.R., Stout, J.R., Schmitz, S., Collins, R. Kalman, D.S., Antonio, J., & Kreider, R.B. (2013). International society of sports nutrition position stand: energy drinks. *J. Int. Soc. Sports Nutr,* 10, 1. https://doi.org/10.1186/1550-2783-10-1.

Chen, W., Guo, J., Zhang, Y., & Zhang, J., (2016). The beneficial effects of taurine in preventing metabolic syndrome*. Food Funct,* 7(4), 1849–1863.

Correa, M., SanMiguel, N., López-Cruz, L., Carratalá-Ros, C., Olivares-García, R., & Salamone, J.D.(2018). Caffeine Modulates Food Intake Depending on the Context That Gives Access to Food: Comparison with Dopamine Depletion. *Front. Psychiatry,* 9, 411. doi:10.3389/fpsyt.2018.00411.

De Sanctis, V., Soliman, N., Soliman, A.T., Elsedfy, H., Di Maio, S., El Kholy, M., & Fiscina, B.(2017). Caffeinated energy drink consumption among adolescents and potential health consequences associated with their use: a significant public health hazard. *Acta Biomed,* 88(2): 222–231. DOI: 10.23750/ABM. v88i2.6664.

Dillon, P., Kelpin, S., Kendler, K., Thacker, L., Dick D., & Svikis, D. (2019). Gender Differences in Any-Source Caffeine and Energy Drink Use and Associated Adverse Health Behaviors. *Caffeine Adenosine Res,* 9(1), 12–19. DOI: 10.1089/caff.2018.0008.

Driescher, N., Joseph, D. E., Human, V. R., Ojuka, E., Cour, M., Hadebe, N., Bester, D., Marnewick, J. L., Lecour, S., Lochner, A., & Essop, M. F. (2019). Corrigendum to "The impact of sugar-sweetened beverage intake on rat cardiac function" [Heliyon 5 (3) (March 2019) e01357]. *Heliyon*, 5(5), e01592. https://doi.org/10.1016/j.heliyon.2019.e01592.

Duchan, E., Patel, N.D., & Feucht, C. (2010). Energy drinks: a review of use and safety for athletes. *Phys. Sportsmed,* 38,171–179. DOI: 10.3810/psm.2010.06.1796.

El-Kashoury, A. A., Salama, A. F., Selim, A. I., & Mohamed, R. A.(2010).Chronic exposure of dicofol promotes reproductive toxicity in male rats. *Life Sci,* 7(3), 5–19.

Esteves, S.C. (2015). Male infertility due to spermatogenic failure: current management and future perspectives. *Anim. Reprod,* 12(1),62–80.

FAO (Food and Agriculture Organization of the United Nations)(1994. Definition and classification of commodities. 3. Sugar crops and sweeteners and derived products. Retrieved from http://www.fao.org/es/faodef/faodefe.htm.

Fields, S.K., MacDonald, J., Joseph, A.M., Wold, L.E., Collins, C.L., & Comstock, R.D.(2015). Consumption of sports and energy drinks by high school athletes in the United States: a pilot study. *Beverages,* (1), 218–224. https://doi.org/10.3390/ beverages1030218.

Friis, K., Lyng, J.I., Lasgaard, M., & Larsen, F.B. (2014). Energy drink consumption and the relation to socio-demographic factors and health behaviour among young adults in Denmark. A population-based study. *Eur. J. Public Health,* 24(5), 840–844. doi:10.1093/eurpub/cku003.

Gaby, A.R. (2005). Adverse effects of dietary fructose. *Altern. Med. Rev,* 10,294–306. http://archive.foundationalmedicinereview.com/publications/10/4/294.pdf.

Gatsing, D., Nkeugouapi, C.F.N., Nkah, B.F.N., Kuiate, J.R., & Tchouanguep, F.M.(2010). Antibacterial activity, bioavailability and acute toxicity evaluation of the leaf extract of Alchorneacordifolia (Euphorbiaceae). *Int. J. Pharm,* 6, 173–182.doi: 10.3923/ijp.2010.173.182.

Govardhan, N.A., & Changamma C. (2014). Antifertility efficacy of bitter leaf stalk extract in male albino rats. *J. Adv. Res,* 2(3), 97–102.

Graneri, L.T., Mamo, J.C.L., D'Alonzo, Z., Lam, V., & Takechi, R. (2021). Chronic Intake of Energy Drinks and Their Sugar Free Substitution Similarly Promotes Metabolic Syndrome. *Nutrients,* 13(4), 1202. https://doi.org/10.3390/nu13041202.

Harpaz, E., Tamir, S., Weinstein, A., & Weinstein, Y. (2017). The effect of caffeine on energy balance. *J. Basic Clin. Physiol. Pharmacol,* 28(1), 1–10. doi:10.1515/jbcpp-2016-0090.

Heckman, M.A., Weil, J., & Mejia, E.G.(2010). Caffeine (1, 3, 7-trimethylxanthine) in foods: A comprehensive review on consumption, functionality, safety, and regulatory matters. *J. Food Sci,* 75,75–87. doi: 10.1111/j.1750-3841.2010.01561.x.

Jaffé, W.R. (2015). Nutritional and functional components of noncentrifugal cane sugar: A compilation of the data from the analytical literature. *J. Food Compost. Anal,* 43, 194–202. doi:10.1016/j.jfca.2015.06.007.

Kamal, R., Yadav, R., & Sharma, J. D. (1993). Efficacy of the steroidal fraction of fenugreek seed extract on fertility of male albino rats. *Phytother. Res,*7(2), 134–138.

Karvonen, M.J., & Malm, M. (1955). Colorimetric determination of fructose with indol. *Scand. .J Clin. Lab. Invest*, 7,305–307.

Kawasaki, T., Kashiwabara A., Sakai T., Igarashi K., Ogata N., & Watanabe H. (2005). Long-term sucrose-drinking causes increased body weight and glucose intolerance in normal male rats. *Br. J. Nutr,* 93, 613–618.

Keaver, L., Gilpin, S., Fernandes da Silva, J.C., Buckley, C., & Foley-Nolan, C. (2017). Energy drinks available in Ireland: a description of caffeine and sugar content. *Public Health Nutr,* 20,1534–1539. DOI: 10.1017/S1368980017000362.

Kumar, A., & Singh, S. (2019). The benefit of Indian jaggery over sugar on human health. In: *Dietary Sugar, Salt, and Fat in Human Health* pp. 1-12. DOI: 10.1016/B978-0-12-816918-6.00016-0.

Kuramori, C., Hase, Y., Hoshikawa, K., Watanabe, K., Nishi, T., Hishiki, T., Soga, T., Nashimoto, A., Kabe, Y., Yamaguchi, Y., Watanabe, H., Kataoka, K., Suematsu, M., & Handa, H. (2009). Mono-(2-ethylhexyl) phthalate targets glycogen debranching enzyme and affects glycogen metabolism in rat testis. *Toxicol. Sci,* 109(1), 143-151. https://doi.org/10.1093/toxsci/kfp041.

Lavin, J.H., French, S.J. , Ruxton, C.H., & Read, N.W. (2002). An investigation of the role of oro-sensory stimulation in sugar satiety. *Int. J. Obes. Relat. Metab. Disord*, 26, 384-388.

Munteanu C., Uţiu, I., Roşioru, C.L., & Lang, C. (2014). Chronic administration of red bull affects blood parameters in rats. *Studia UBB Biologia,* 2, 89-98.

Nath, A., Dutta, D., Kumar, P., & Singh, J.P. (2015). Review on recent advances in value addition of jaggery based products. *J. Food Process. Tech,* 6, 4. doi: 10.4172/2157-7110.1000440.

Nallella, K.P., Sharma, R.K., Aziz, N., & Agarwal, A. (2006). Significance of sperm characteristics in the evaluation of male infertility. *Fertil. Steril,* 85(3),629-634. doi: 10.1016/j.fertnstert.2005.08.024.

Patel, M., Sharma, R.J., Kumar,S., Kumar, A., Tiwari, D. P., & Kumar, R. (2011). Effect of feeding different levels of jaggery filter cake on blood biochemical and mineral profile in Yorkshire pigs. *Indian J. Anim. Sci,* 81 (2), 180.

Rao, G.P., & Singh, P. (2022). Value Addition and Fortification in Non-Centrifugal Sugar (Jaggery): A Potential Source of Functional and Nutraceutical Foods. *Sugar Tech,* 24, 387–396. https://doi.org/10.1007/s12355-021-01020-3.

Rebourcet, D., Mackay, R., Darbey, A., Curley, M.K., Jørgensen, A., Frederiksen, H., Mitchell, R.T., O'Shaughnessy, P.J., Nef, S., & Smith, L.B. (2020). Ablation of the canonical testosterone production pathway via knockout of the steroidogenic enzyme HSD17B3, reveals a novel mechanism of testicular testosterone production. *FASEB J,* 34(8),10373-10386. doi: 10.1096/fj.202000361R.

Reissig, C.J., Strain, E.C., & Griffiths, R.R. (2009). Caffeinated energy drinks-a growing problem. *Drug Alcohol Depend,* 99(1-3),1-10. doi: 10.1016/j.drugalcdep.2008.08.001.

Schuchowsky, E., Schaefer, D., Salvador, R.A., do Nascimento, A.E., Til, D., Senn, A.P., & Amaral, V.L.L.(2017). Effects of energy drinks on biochemical and sperm parameters in Wistar rats. *Nutrire,* 42(1), 1-7. doi:10.1186/s41110-017-0047-9.

Tappy, L, & Lê K.A. (2010). Metabolic effects of fructose and the worldwide increase in obesity. *Physiol. Rev*, 90,23-46, 10.1152/physrev.00019.2009.

Togo, J., Hu, S., Li, M., Niu, C., & Speakman, J. R. (2019). Impact of dietary sucrose on adiposity and glucose homeostasis in C57BL/6J mice depends on mode of ingestion: liquid or solid. *Mol. Metab*, 27, 22–32. https://doi.org/10.1016/j.molmet.2019.05.010.

Zhang, Z., Yang, Z., Zhu, B., Hu, J., Liew, C.W., Zhang, Y., & Stanton, R.C. (2012). Increasing glucose 6-phosphate dehydrogenase activity restores redox balance in vascular endothelial cells exposed to high glucose. *PLoS ONE,* 7(11), e49128. doi.org/10.1371/journal.pone.0049128.

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Inhibitory potential of some selected essential oils and their main components on the growth and quorum-sensing based pigment production of *Serratia marcescens*

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Abstract. In this study the antibacterial potential of ten essential oils (EOs) and their main compounds against the development and quorum sensing (QS) mechanisms of the opportunistic bacterium *Serratia marcescens* was determined. The growth and QS inhibitory effect was evaluated by paper disc diffusion assay. The effect of EOs and components on QS-regulated prodigiosin biosynthesis was also studied.

The results of our study indicated that some of the investigated EOs influenced the development and the QS-based activity of *S. marcescens*. Oregano and thyme oils showed the most pronounced antibacterial effect and had the strongest anti-QS potential. From the main oil compounds the phenolics, eugenol, carvacrol and thymol, proved to be efficient growth inhibitors. While eugenol and carvacrol had also a strong negative influence on AHL-mediated QS-systems in low concentrations, thymol was not effective in QS inhibition.

Keywords: essential oils, quorum-sensing, prodigiosin.

Introduction

The bacteria are capable to socially interact by quorum-sensing (QS) mechanism, which is a density-dependent cell-to-cell communication. This unique bacterial communication system involves the synthesis of diffusible

autoinducer molecules, which permit unicellular bacteria to regulate gene expression in order to synchronize their behaviour in accordance with population density. In Gram-negative bacteria the most common QS signal molecules are acylated homoserine lactones (AHLs) (de Kievit and Iglewski, 2000; Waters and Bassler, 2005; Coulthurst *et al.*, 2006; Khanafari *et al*., 2006; Van Houdt *et al*., 2007; Szabó *et al.*, 2010; Robson *et al.*, 2014).

The density-dependent QS signaling systems control diverse physiological functions in bacteria, such as biofilm formation, virulence factor and extracellular polysaccharides synthesis, secondary metabolite synthesis, swimming motility and bacterial resistance. Therefore, by controlling or inhibiting QS processes it could be possible to influence the microbial processes regulated by this mechanism (Brown and Johnstone, 2001; Annous *et al.*, 2009).

Serratia marcescens is a ubiquitous environmental Gram-negative bacterium which is well known for the production of a characteristic red pigment, prodigiosin. The biosynthesis of prodigiosin is directed by the *pig* gene cluster and is regulated by AHL-mediated QS mechanism (Thomson *et al*., 2000; Haddix *et al.*, 2008).

Nowadays the growing interest in study of *S. marcescens* is related to some important findings. First, prodigiosin produced by many *Serratia marcescens* strains has been identified as having extremely broad antimicrobial activities and potent immunosuppresive, proapoptic and anticancer properties (Khanafari *et al.*, 2006; Samrot *et al.*, 2011; Kamble and Hiwarale, 2012; Do and Nguyen, 2014). Due to the various applications of prodigiosin it would be important to understand the effect of different factors interfering with QS-regulated prodigiosin production. On the other hand, *S. marcescens* is an opportunistic pathogen, which is responsible for an increasing number of nosocomial infections and was repeatedly isolated from respiratory and urinary tract clinical samples; *S. marcescens* is also the causative agent of conjunctivitis in contact lens wearers (Robson *et al*., 2014). Nosocomial infections due to these clinical isolates are frequently problematic because the pathogens are commonly multidrug resistant (Morohoshi *et al.*, 2007). *Serratia* strains have been also reported to be one of the causative agents of food spoilage (Turgis *et al.*, 2012). Thus, it is necessary to develop new treatment or control possibilities for *Serratia* infections.

Essential oils (EOs) are aromatic and volatile hydrophobic mixtures, originated from the plant secondary metabolism. EOs are obtained from different plant parts (leaves, flowers, seeds) mainly by steam distillation and contain more than 50 active components in different proportions (Bakkali *et al.*, 2008).

Due to their well known antimicrobial potential, these vegetal constituents get an increasing attention in medicine and pharmaceutical industry, in aroma therapy and cosmetic industry, as well as in biocontrol techniques and food industry.

Natural EOs have the advantage that they are more ecofriendly, inhibiting not only the growth, but also the microbial density-dependent cell-to-cell communication systems, so the development of the microbial resistance to their active compounds is much less likely (Kerekes *et al.*, 2013).

The aim of this study was to investigate the effect of some selected essential oils and their main components on the development of *Serratia marcescens* as well as on the QS-controlled pigment formation processes.

Materials and methods

Bacterial Strains, Media, and Culture Conditions

The *Serratia marcescens* SZMC 0567 strain used in the experiments was from Szeged Microbiological Collection, maintained by the Department of Microbiology of the University of Szeged, Hungary. The bacterium strain was grown on peptone glycerol agar and peptone glycerol broth, respectively.

Essential Oils and Major Components

The investigated essential oils were marjoram (*Origanum majorana*), lemon (*Citrus lemon*), clary sage (*Salvia sclarea*), juniper (*Juniperus communis*), cinnamon (*Cinnamomum zeylanicum*), thyme (*Thymus vulgaris*), lavender (*Lavandula officinalis*), oregano (*Origanum vulgare*), clove (*Syzygium aromaticum*) and summer savory (*Satureja hortensis*). The main components of the tested essential oils (eugenol, thymol, carvacrol, limonene, β-o-cimene, linalool, α-thujone, α-pinene) were also investigated.

Detection of Growth and Quorum-Sensing Inhibition by Diffusion Paper Assay

The liquid culture of *S. marcescens* with a density of 10^8 cfu ml^{-1} was spread in Petri dishes on the surface of peptone glycerol agar. EOs and their main components were applied in different quantities (1, 3, 5 and 8 µl) on sterile paper discs placed on the surface of the inoculated media. After incubation at 37 ^oC for 24-48 h the effect of EOs and compounds against the

development of bacteria was determined by measuring the inhibition zone around the paper discs, whereas QS-inhibition effect was determined by estimating the colourless zone developed behind the inhibition zone.

Determination of Prodigiosin Synthesis in the Presence of EOs and Major Compounds

From the *S. marcescens* suspension (OD 0,506 at 620 nm) 100 µl ml^{-1} was inoculated in peptone glycerol broth, containing different quantities of essential oils or their main compounds (1, 3, 5, 8, 10, 13, 15, 18, 20, 23, 26 µl ml^{-1} culture medium). For the extraction of prodigiosin, bacterial suspensions incubated for 1 week were used, where there was an obvious evidence of prodigiosin production because the colour of the suspension turned from yellow to red. The cells were harvested by centrifugation at 10 000 rpm for 10 min. The supernatant was discarded and the pellet was resuspended in acidified ethanol (4% of 1M HCl in 96 ml ethanol). The mixture was centrifuged again, and the absorbance values of the supernatant containing the extracted prodigiosin was determined at 499 nm, where the prodigiosin absorbs maximally (Slater *et al.*, 2003).

Isolated prodigiosin was estimated using the following formula (Mekhael and Yousif, 2009):

$$\text{Prodigiosin unit/cell} = \frac{[OD499 - (1.381 * OD620)] * 1000}{OD620}$$

OD499 – pigment absorbance; OD620 – bacterial cell absorbance;
1.381 – constant

Six parallel measurements were made for each variant. The control underwent the whole procedure except for the oil/component treatment.

Statistical Analysis and Data Processing

For experimental data evaluation, R statistical analysis program was used. The comparison of different treatment groups was performed using one-way ANOVA and Tukey test, by evaluating the significant differences ($P < 0.01$) within the groups. For some data the Kruskal-Wallis non-parametric test was used.

Results

Growth and QS Inhibitory Effect of EOs and their Main Components

The results regarding growth and QS inhibition of essential oils are shown in Table 1. Five of the EOs tested (lemon, juniper, clary sage, marjoram and lavender) had no influence on the growth and QS communication mechanisms of *S. marcescens*, even if we have applied them in increasing concentrations. Their major components (limonene, α- pinene, α- thujone, linalool, β- ocimene) were also ineffective in the control of *S. marcescens* (Tabs. 1 and 2).

Table 1. The effect of essential oils on the growth and QS of *S. marcescens* (paper disc diffusion assay)

	Growth inhibition zone (mm)					**Quorum-sensing inhibition zone (mm)**				
Essential oils	1 µl	2 µl	3 µl	5 µl	8 µl	1 µl	2 µl	3 µl	5 µl	8 µl
cinnamon	20	24.6	26	27.3	29	5.6	4.6	4	-	-
clove	15.6	20	16.3	29.6	31	-	-	-	9.6	10.3
thyme	17.3	18	22.3	40.3	38.6	-	-	1.6	14.3	14.7
oregano	18	23	21.6	40	41.6	-	-	-	11.3	14.8
savory	12.3	16.6	18	23.4	31	-	-	-	-	-
lemon	-	-	-	-	-	-	-	-	-	-
juniper	-	-	-	-	-	-	-	-	-	-
clary sage	-	-	-	-	-	-	-	-	-	-
marjoram	-	-	-	-	-	-	-	-	-	-
lavander	-	-	-	-	-	-	-	-	-	-

Table 2. The effect of the essential oil major components on the growth and QS of *S. marcescens* (paper disc diffusion assay)

Aromatic compounds	**Growth inhibition halo (mm)**					**Quorum-sensing inhibition halo (mm)**				
	1 µl	2 µl	3 µl	5 µl	8 µl	1 µl	2 µl	3 µl	5 µl	8 µl
eugenol	16	27.6	30.3	28.6	29	3	11.6	12.6	10	10
carvacrol	20.6	27.6	34	38.6	36.6	4	7.3	10.6	10.6	10
thymol	15.3	16.3	19.3	19.3	20.3	-	-	-	-	-
limonene	-	-	-	-	-	-	-	-	-	-
α-pinene	-	-	-	-	-	-	-	-	-	-
α-thujone	-	-	-	-	-	-	-	-	-	-
linalool	-	-	-	-	-	-	-	-	-	-
β-ocimene	-	-	-	-	-	-	-	-	-	-

One can observe the inhibitory capacity of cinnamon, clove, thyme, oregano and summer savory oils on the growth of *S. marcescens*. Cinnamon oil had a good inhibitory potential on the growth of *S. marcescens* even in the lowest concentration; by increasing the oil concentrations there were no significant differences between the samples (Fig.1).

In the case of some oils tested (clove, thyme, oregano and summer savory) the inhibitory effect against the bacterial growth was significantly pronounced as the concentration increased. At the highest quantity (8µl) the diameter of growth inhibition halo produced by all of these oils exceeded the halo due to cinnamon oil, where the inhibitory effect was not concentration-dependent (Fig. 1). The most pronounced inhibition was detected in the cases of oregano and thyme oils.

As Tab. 1 shows, the cinnamon oil slightly interfere with QS communication systems, as the inhibitory potential of QS was detected only at lower quantities, while clove, thyme and oregano EOs had anti-QS potential only in higher concentrations. The most pronounced inhibition of QS was observed in the case of oregano at 8 µl. Even if the savory EO proved to be a good inhibitor of the bacterial growth, it did not show inhibitory effect on QS-systems in the concentrations used (Tab. 1).

Some of the major aromatic compounds tested (eugenol, carvacrol and thymol) had negative effect against the development of *S. marcescens* (Tab. 2). Comparing the effect of components on the growth of *S. marcescens* no significant differences have been observed at low amounts, but as the applied quantities increased the inhibitory potential differ considerably (Fig. 2).

Eugenol, which is the main component of cinnamon and clove oils, had a strong quantity-dependent antibacterial effect, interfering not only with the growth, but also with the QS regulation systems of *S. marcescens*. Carvacrol, found in oregano, thyme and savory oils, proved to be the best growth inhibitor and interfere with QS-based communication systems, the inhibitory potential increased significantly at higher quantities. Surprisingly, in case of thymol, the another aromatic compound of the mentioned EOs, a good anti-growth effect was observed, but we could not detect any anti-QS potential (Tab. 2).

These findings suggest that the growth-inhibitory effect of oregano, thyme and savory oils is due to carvacrol and thymol, while the negative effect of oregano and thyme EOs on QS systems is related to carvacrol, found in considerable amounts (up to 60%) in these EOs. Comparing the growth-reducing effect of parent EOs with their main components in the case of eugenol we have found a same antimicrobial potential, obtaining a significantly stronger effect only between eugenol and clove oil at the concentration of 3 µl (Fig. 3).

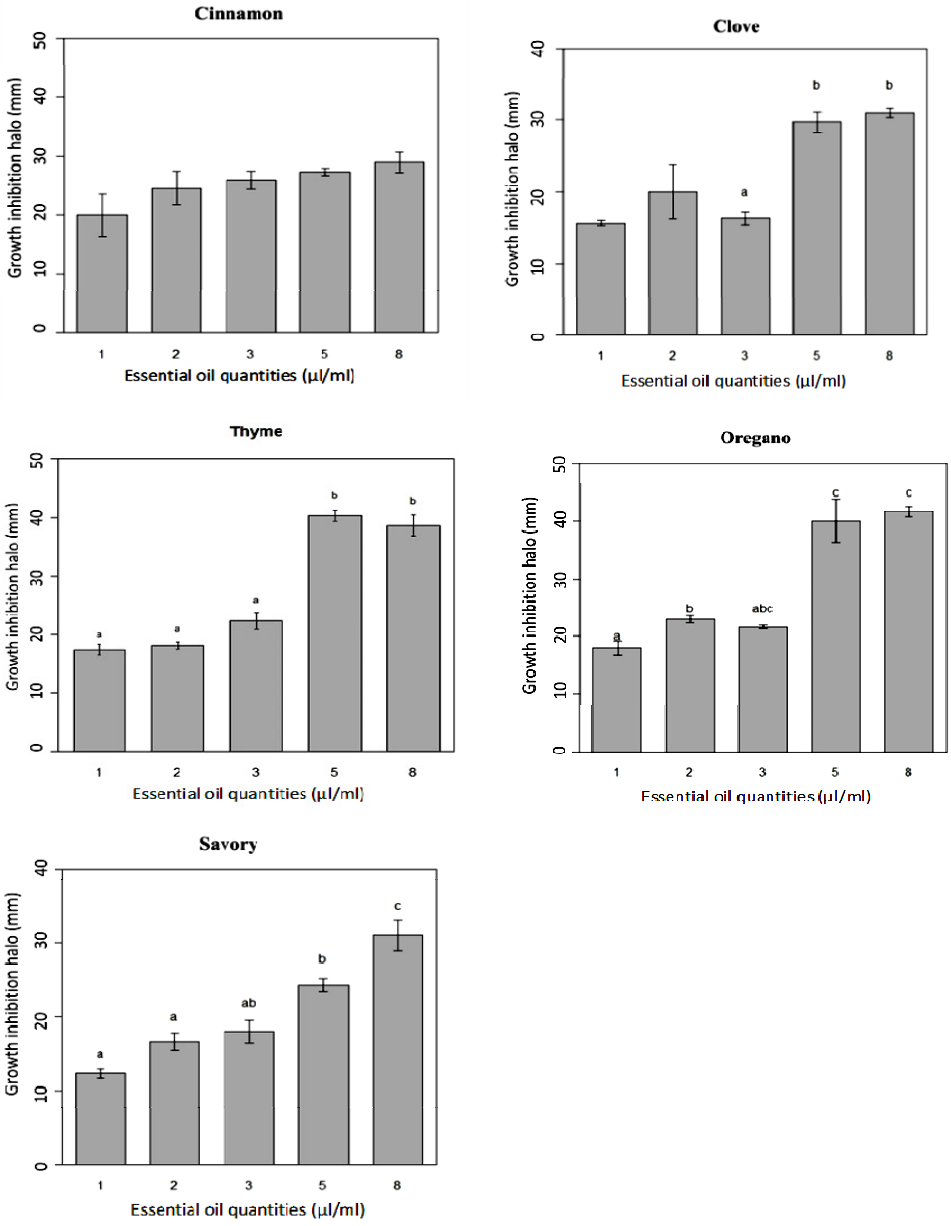

Figure 1. The inhibitory potential of the EOs studied. Significant changes are represented by different letters, while the same letters indicate no significance. Double or triple letters indicate that the given variant does not differ significantly from the variants marked with one or the other letters.

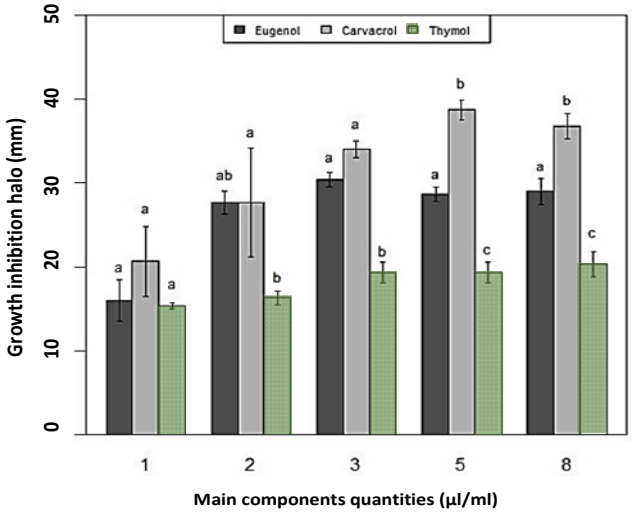

Figure 2. The growth inhibitory potential of some oil components. Different letters represent significant changes, while the same letters indicate no significance. Double letters indicate that the given variant does not differ significantly from the variants marked with one or the other letter.

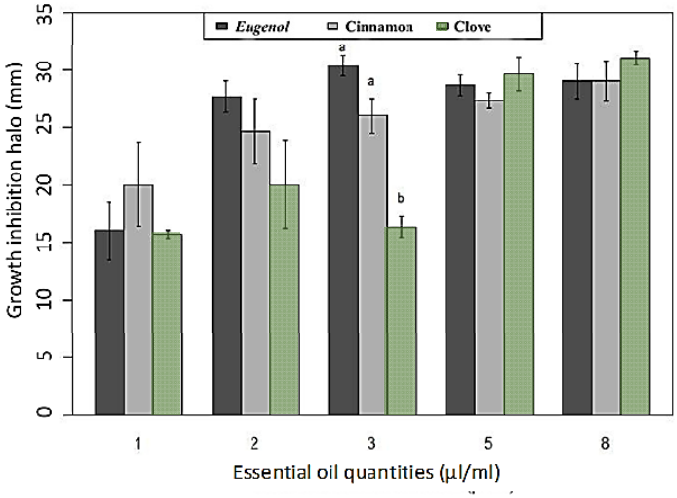

Figure 3. Comparison of the anti-growth potential of eugenol and parent EOs. Significant changes are represented by different letters, while the same letters indicate no significance. Double letters indicate that the given variant does not differ significantly from the variants marked with one or the other letter.

At large, the carvacrol presented also the same antimicrobial effectiveness than the parent EOs, having a considerably better inhibitory effect than thyme and oregano oils only when was applied in a quantity of 3 µl (Fig. 4).

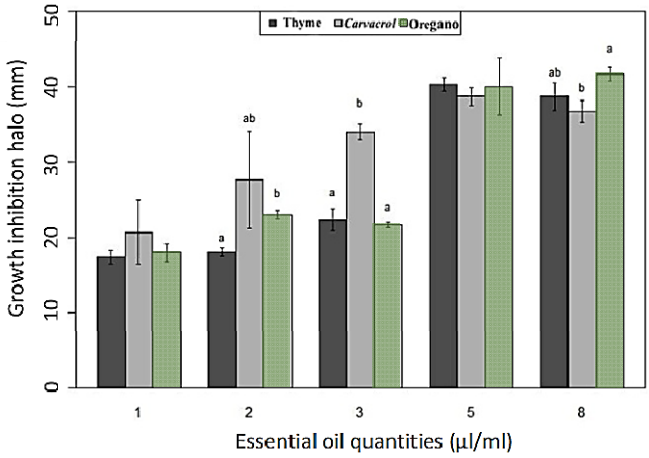

Figure 4. Comparison of the anti-growth potential of carvacrol and parent EOs. Significant changes are represented by different letters, while the same letters indicate no significance. Double letters indicate that the given variant does not differ significantly from the variants marked with one or the other letter.

Effect of EOs and their Components on QS-regulated Prodigiosin Synthesis

As we have expected, the EOs which had inhibitory potential on the growth and QS regulation systems of *S. marcescens*, demonstrated by diffusion methods, significantly decreased the rate of prodigiosin production. In the case of cinnamon and oregano oils we could observe the same tendency in the inhibition of pigment synthesis, both oils had a strong negative influence even in the smallest quantities. There were no significant differences between the samples treated with increased oil concentrations. In contrast, thyme and clove EOs had a significant inhibitory effect against QS based prodigiosin production, the effectiveness of oils increased in higher concentrations (Fig. 5).

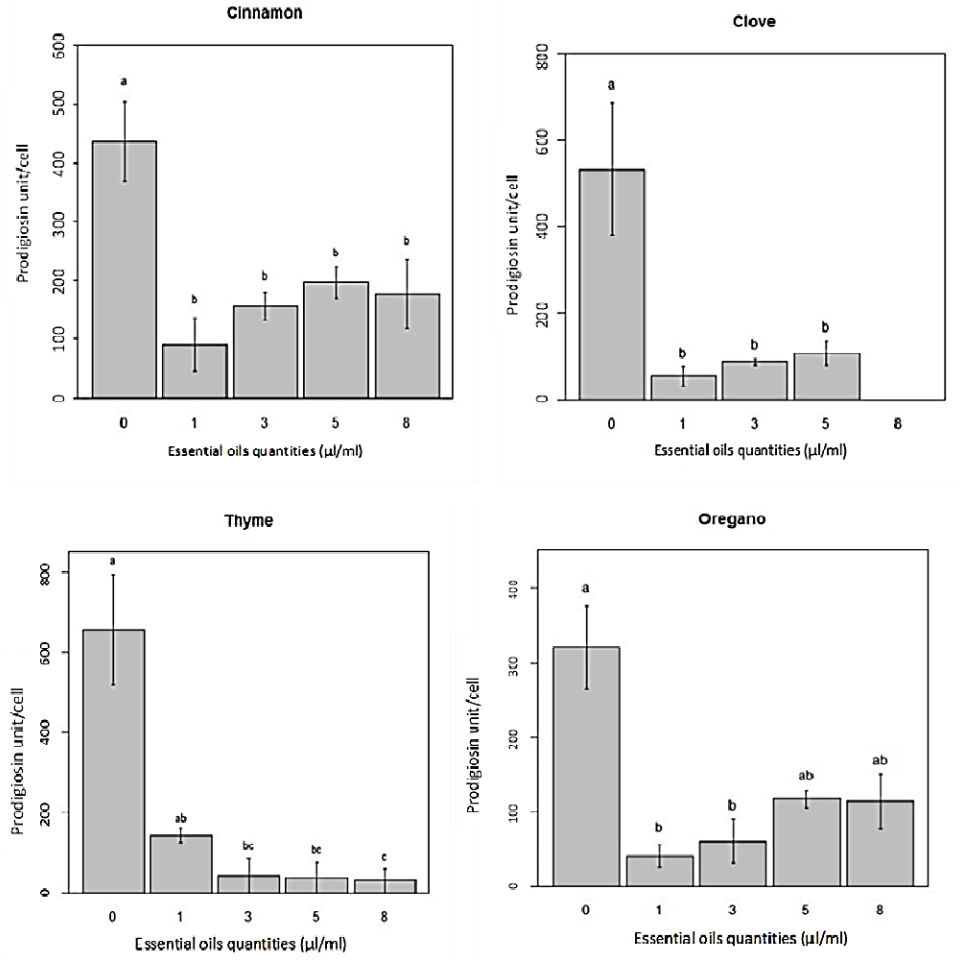

Figure 5. Effect of essential oils on the prodigiosin biosynthesis of *S. marcescens*. Different letters represent significant changes, while the same letters indicate no significance. Double letters indicate that the given variant does not differ significantly from the variants marked with one or the other letter.

The major aromatic compounds of the EOs tested also influenced the QS-regulated prodigiosin biosynthesis of *S. marcescens*. As shown in Fig. 6, in the presence of eugenol the pigment production decreased significantly, but the increase of the applied quantities has not correlated with the intensification

of the prodigiosin biosynthesis inhibitory effect. The carvacrol had a pronounced quantity independent negative effect on the QS-mediated pigment production of *S. marcescens*, so it proved to be a very strong QS inhibitor. Generally, these results are in agreement with that found by paper disc assay.

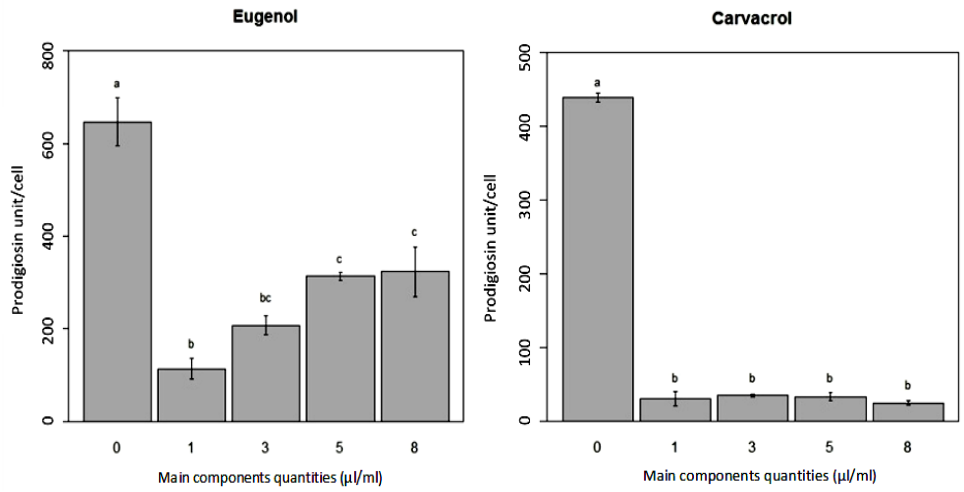

Figure 6. Effect of eugenol and carvacrol on the rate of prodigiosin biosynthesis. Different letters represent significant changes, while the same letters indicate no significance. Double letters indicate that the given variant does not differ significantly from the variants marked with one or the other letter.

Discussion

Quorum sensing systems of *S. marcescens* act as a global regulator of almost all the virulence factors, sliding motility and biofilm formation (Annous *et al.*, 2009; Kumar *et al.*, 2016). The biosynthesis of the red prodigiosin pigment is also under QS regulation, therefore, this pigment-producing bacterium can be used as a biosensor in QS regulation investigations. Since the QS regulation directly accords to its pathogeny, targeting QS systems could provide a new strategy to control the virulence of this bacterium (Bakkiyaray *et al*., 2012).

Essential oils and their aromatic compounds are assumed to not result in the development of the resistance in bacterial strains. Instead, this natural products attenuate the expression of genes responsible for pathogenesis by interferring with bacterial communication systems (Packiavathy *et al.*, 2012).

In general terms, the essential oils influence the integrity of the cell wall and cytoplasmic membrane, determining the loss of some cell compounds, that finally leads to death of the microbial cells.

Our results suggest that EOs containing phenolic compounds such as carvacrol, thymol and eugenol proved to be the strongest inhibitors of *Serratia marcescens*. These data are in agreement with other studies, that confirm that these compounds have a dose dependent inhibitory effect on enteroinvasive *Escherichia coli* and other Gram-negative bacteria (Kim *et al.*, 1995; Cosentino *et al*., 1999; Dušan *et al.*, 2006; Gill and Holley, 2006). The phenolic components determine the release of lipopolysaccharides from the outer membrane and cause the disintegration of the cell wall. The inhibitory action is also explained by the increase of the cell membrane permeability to ATP and by the ATPase inhibiting activity of these compounds. In addition eugenol has a role in the inhibition of specific cellular processes and enzymes (Burt, 2004; Gill and Holley, 2006; Seydim and Sarikus, 2006; Kerekes *et al.*, 2013).

The EOs containing cyclic terpenes and terpene alcohols as main constituents have no effect on growth and QS-based prodigiosin biosynthesis of *S. marcescens*.

The antimicrobial effect of the EOs usually is due to the interaction between all the constituents and not only to a single component (Lis-Balchin and Deans, 1997; Mourey and Canillac, 2002), however there are some evidences that in some cases the components are better inhibitors than EOs and the use of a single compound is sufficient to treat bacterial biofilms (Kerekes *et al*., 2013). According to our findings, the effect of the main compounds on the growth of *S. marcescens* differ not significantly from those of essential oils, suggesting that the EOs with phenolic compounds or only their major bioactive components applied in suitable quantities could be efficient biological alternatives in controlling the growth and virulence determining processes of *S. marcescens.*

Conclusions

Some of the investigated essential oils influenced significantly the development and the QS-based pigment production of opportunistic *S. marcescens*. Oregano and thyme oils showed the most pronounced antibacterial effect and had the strongest anti-QS potential. The phenolic oil components, such as eugenol, carvacrol and thymol, proved to be efficient growth inhibitors.

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References

Annous, B.A., Fratamico, P.M. & Smith, J.L. (2009). Quorum sensing in biofilms*:* why bacteria behave the way they do. *J. Food Sci.*, *74*, 24-37.

Bakkiyaray, D., Sivasankar, C. & Pandian, S.K. (2012). Inhibition of quorum sensing regulated biofilm formation in *Serratia marcescens* causing nosocomial infections. *Bioorg. Med. Chem. Lett.*, *22*, 3089-3094.

Bakkali, F., Averbeck, S., Averbeck, D. & Idaomar, M. (2008). Biological effect of essential oils – a review. *Food Chem. Toxicol.*, *46*, 446-475.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods – a review. *Internat. J. Food Microbiol*., *94*, 223-253.

Brown, S.P. & Johnstone, R.A. (2001). Cooperation in the dark: signaling and collective action in quorum-sensing bacteria. *Proc. Biol. Sci.*, *268*, 961–965.

Cosentino, S., Tuberoso, C.I.G., Pisano, B., Satta, M., Mascia, V., Arzedi, E. & Palmas, F. (1999). *In vitro* antimicrobial activity and chemical composition of Sardinian Thymus essential oils. *Lett. Appl. Microbiol.*, *29*, 130-135.

Coulthurst, S.J., Williamson, N.R., Harris, A.K.P., Spring, D.R. & Salmond, G.P.C. (2006). Metabolic and regulatory engineering of *Serratia marcescens* mimicking phage-mediated horizontal acquisition of antibiotic biosynthesis and quorum-sensing capacities. *Microbiology*, *152*, 1899-1911.

de Kievit, T.R. & Iglewski, B.H. (2000). Bacterial quorum sensing in pathogenic relationships. *Infect. Immun*., *68*, 4839-4849.

Do, H.N.A. & Nguyen, T.H.K. (2014). Studies on the prodigiosin production from *Streptomyces coelicolor* in liquid media using heated *Lactobacillus rhamnosus*. *J. Applied Pharmaceut. Sci.*, *4*, 21-24.

Dušan, F., Sabol, M., Domaracká, K. & Bujňáková, D. (2006). Essential oils – their antimicrobial activity against *Escherichia coli* and effect on intestinal cell viability. *Toxicol. In Vitro*, *20*, 1435-1445.

Gill, A.O. & Holley, R.A. (2006). – Disruption of *Escherichia coli*, *Listeria monocytogenes* and *Lactobacillus sakei* cellular membranes by plant oil aromatics. *Internat. J. Food Microbiol.*, *108*, 1-9.

Haddix, P.L., Jones, S., Patel, P., Burnham, S., Knights, K., Powell, J.N. & LaForm, A. (2008). Kinetic analysis of growth rate, ATP, and pigmentation suggests an energy-spilling function for the pigment prodigiosin of *Serratia marcescens*. *J. Bacteriol.*, *190*, 7453-7463.

Kamble, K.D., Hiwarale, V.D. (2012). Prodigiosin production from *Serratia marcescens* strains obtained from farm soils. *Internat. J. Environ. Sci.*, *3*, 631-638.

Kerekes, E.B., Deák, É., Takó, M., Tserennadmid, R., Petrovits, T., Vágvölgyi, C. & Krisch, J. (2013). Anti-biofilm forming and anti-quorum sensing activity of selected essential oils and their main components on food-related micro-organism. *J. Appl. Microbiol.*, *115*, 933-942.

Khanafari, A., Assadi, M.M. & Fakhr, F.A. (2006). Review of prodigiosin, pigmentation in *Serratia marcescens. J. Biol. Sci.*, *6(1)*, 1-13.

Kim, J., Marshall, M.R. & Wei, C.I. (1995.) Antibacterial activity of some essential oil components against five foodborne pathogens. *J. Agricult. Food Chem.*, *43*, 2839-2845.

Kumar, J.S., Umesha, S., Prasad, K.S. & Niranjana, P. (2016). Detection of quorum sensing molecules and biofilm formation in *Ralstonia solanacearum*. *Curr. Microbiol.*, *72*, 297-305.

Lis-Balchin, M. & Deans, S.G. (1997). Bioactivity of selected plant essential oils against *Listeria monocytogenes*. *J. Appl. Microbiol.*, *82*, 759-762.

Mekhael, R. & Yousif, S.Y. (2009). The role of red pigment produced by *Serratia marcescens* as antibacterial and plasmid curing agent. *J. Duhok Univ.*, *12*, 268-274.

Morohoshi, T., Shiono, T., Takidouchi, K., Kato, M., Kato, N., Kato, J. & Ikeda, T. (2007). Inhibition of quorum sensing in *Serratia marcescens* AS-1 by synthetic analogs of N-acyl-homoserine lactone. *Appl. Environ. Microbiol.*, *73*, 6339-6344.

Mourey, A. & Canillac, N. (2002). Anti-*Listeria monocytogenes* activity of essential oils components of conifers. *Food Contrib.*, *13*, 289-292.

Packiavathy, I.A.S.V., Agilandeswari, P., Musthafa, K.S., Pandian, S.K. & Ravi, A.V. (2012). Antibiofilm and quorum sensing inhibitory potential of *Cuminum cyminum* and its secondary metabolite methyl eugenol against Gram negative bacterial pathogens. *Food Res. Internat.*, *45*, 85-92.

Robson, E., Yan-Lue, L., Kok-Keng, T., Wai-Fong, Y. & Kok-Gan, C. (2014.) *Quorum sensing* activity of *Serratia fonticola* strain RB-25 isolated from an ex-landfill site. *Sensors*, *14*, 5136-5146.

Samrot, A.V., Senthilkumar, C.K. & Kumar, N.G. (2011). Optimization of prodigiosin production by *Serratia marcescens* SU-10 and evaluation of its bioactivity. *Internat. Res. J. Biotechnol.*, *2*, 128-133.

Seydim, A.C. & Sarikus, G. (2006). Antimicrobial activity of whey protein based edible films incorporated with oregano, rosemary and garlic essential oils. *Food Res. Internat.*, *39*, 639-644.

Slater, H., Crow, M., Everson, L. & Salmond, G.P. (2003). Phosphate availability regulates biosynthesis of two antibiotics, prodigiosin and carbapenem, in *Serratia* via both quorum-sensing-dependent and -independent pathways. *Molec. Microbiol.*, *47*, 303–320.

Szabó, M.Á., Varga, G.Z., Hohmann, J. & Schelz, Z. (2010). Inhibition of quorum-sensing signals by essential oils. *Phytother. Res.*, *786*, 782–786.

Thomson, N.R., Crow, M.A., McGowan, S.J., Cox, A. & Salmond, G.P. (2000). Biosynthesis of carbapenem antibiotic and prodigiosin pigment in *Serratia* is under quorum sensing control. *Molec. Microbiol.*, *36*, 539-556.

Turgis, M., Vu, K.D., Dupont, C. & Lacroix, M. (2012). Combined antimicrobial effect of essential oils and bacteriocins against foodborne pathogens and food spoilage bacteria. *Food Res. Internat.*, *48*, 696-702.

Van Houdt, R., Givskov, M. & Michiels, C.W. (2007). Quorum sensing in *Serratia*. *FEMS Microbiol. Rev.*, *31*, 407-424.

Waters, C.M. & Bassler, B.L. (2005). Quorum sensing: cell-to-cell communication in bacteria. *Annu. Rev. Cell Develop. Biol.*, *21*, 319–346.

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Survey of the amphibians in "Fânațele Clujului – Copârșaie", part of the "Dealurile Clujului de Est" (ROSCI0295) Natura 2000 protected area

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Abstract. As habitat loss poses challenge to conservation, it is becoming increasingly important to address questions about the extent to which connectivity between habitat patches is changing, and how this affects the local population of different species in these patches. The objective of our research was to monitor ponds and the pond-breeding amphibian species in a protected area. Therefore, we conducted day and night surveys, and compare the data collected in 2022 with the results of the latest available survey (2019), to simulate the patch occupancy of amphibian species over a 25-year timeframe. We found that combining the species occupancy data collected from both day and night surveys lead to higher patch occupancy values and higher number of registered individuals, compared to data collected only during daytime. The number of ponds decreased from 2019 to 2022, and further habitat loss could result in the disappearance of the local population if the area continues to dry out. Climate and landscape change could be major contributors to habitat loss in the future, therefore, in order to ensure the persistence of these local

populations, we recommend the development of climate and habitat scenarios, and the planning of conservation measures based on these scenarios.

Keywords: amphibian conservation, Transylvanian Plain, SPOM

Introduction

Engaging society to perform nature conservation activities in human-dominated landscapes is a challenge (Robinson, 2006). In Eastern Europe, the proximity of human-nature relationship resulted in landscapes with high cultural and biological diversity (Akeroyd and Page, 2007; Strohbach *et al.*, 2015). In these bioculturally valuable landscapes, the effectiveness of conservation can be enhanced by rethinking the role of humans and reconnecting them to nature by raising awareness to the natural values surrounding them (Grodzińska-Jurczak and Cent, 2011; He *et al.*, 2020, Ives *et al.*, 2018).

Scattered ponds are important parts of the mosaic of cultural landscapes that provide suitable habitats for many species, including amphibians (Calhoun *et al.*, 2017; Cogălniceanu *et al.*, 2012; Hartel and von Wehrden, 2013). From a conservation point of view, these ponds and the amphibian species inhabiting them deserve considerable attention because they are vulnerable elements of the landscape in the era of global change (Blaustein and Kiesecker, 2002; Nori *et al.*, 2015).

Nearly 40% of amphibian species are currently threatened with extinction, yet 53% of these species are distributed mostly outside protected areas (Bolochio *et al.*, 2020). Thus, the conservation of both protected and unprotected landscape features and landscape components that contribute to amphibian conservation is highly important.

According to Curado *et al*. (2011), between 1975 and 2006, the number of amphibian breeding ponds decreased as the grasslands around ponds decreased and the area of crop lands increased. The ponds surveyed in pastures were cattle ponds, which either were filled in or dried up due to land abandonment, resulting in the loss of amphibian breeding habitats, which affected the local amphibian populations (Curado *et al.*, 2011). A recent study showed that changes in a complex, multi-component socio-ecological system led to land abandonment, which resulted in an increase in the area's shrub cover and the drying out of a significant part of the ponds (Erős *et al.*, 2020).

Effective conservation measures require the continuous monitoring of habitats and the knowledge of species abundance and patch occupancy. In the case of amphibians, if data are recorded during multiple field visits, the changes in patch occupancy and probability of species detection can be traced over time (MacKenzie *et al*., 2002; MacKenzie, 2005). These can be important indicators of population and habitat changes (MacKenzie *et al*., 2002). Both field surveys and data analysis are needed to track differences between model and climate or environmental changes (Walls and Gabor, 2019).

The objective of our research was to repeat the survey of ponds and amphibians after three years in a protected area at the periphery of the Transylvanian Plain. Our specific objectives were threefold:

O1. To compare the number and area of ponds, as well as the occurrence of amphibians in ponds with data from 2019 reported by Erős *et al.* (2020).

O2. To construct incidence function models (IFMs) to describe pond profiles that can be occupied by amphibians in the future.

O3. To predict the patch occupancy levels of each amphibian species over a 25-year timeframe using stochastic patch occupancy models.

Materials and methods

Study area

Our study area was within the "Fânațele Clujului – Copârșaie" botanical reserve (46°50′28″ N, 23°38′31″ E), part of the "Dealurile Clujului de Est" Natura 2000 protected area (ROSCI0295). This area was a pasture with steppe vegetation elements grazed by cattle, buffalo and sheep (see Erős *et al.*, 2020). Temporary and permanent-like ponds (i.e., dry out only once in several years, in case of severe drought) were scattered across a 127-ha surface (Fig. 1). Eight amphibian species were reported from this area in the literature: *Bombina variegata*, *Rana dalmatina*, *Pelophylax* kl. *esculentus*, *P. ridibundus*, *Hyla arborea*, *Pelobates fuscus*, *Triturus cristatus*, and an endemic subspecies: *Lissotriton vulgaris ampelensis* (Erős *et al.*, 2020; Sos and Hegyeli, 2015).

Data collection

We conducted field surveys three times in 2022: 12 March, 04 April and 21 May. On each date, we surveyed amphibians at day and night (hereafter referred to as *survey types*). Easily accessible and small-sized ponds (area approx. 10-2000 m^2) were completely surveyed, while larger ponds (area >2000 m^2) were sampled close to the shore (ca. 1-3 m from the shore) at 3-5 sampling points. For some ponds were impossible to sample 3 m from the

shore due to the deep mud. We measured the area of small-sized ponds using metric tape on the field, while the area of larger ponds was measured with GPS device (accuracy < 5 m). We took into account both individuals seen regardless of their stage of development, or heard vocalizing and identified based on their sound. Netting was used to increase the detection of species hiding in the mud, such as *Pelobates fuscus*. We assessed patch occupancy on a binomial scale: 1 if a species was present in a pond at least once during sampling, or 0 if a species was absent during samplings. At each survey occasion one person was responsible for performing the sampling activity, while a field assistant recorded the collected data on the OpenHerpMaps platform (openherpmaps.ro) using the OpenBioMaps mobile application.

Data analysis

To characterise the main changes in the study area in terms of amphibians and their habitats between 2019 and 2020, we compared the area of ponds and their occupancy by amphibians. Therefore, we performed Mann-Whitney U-test, the pond area data being non-normally distributed. We provide median and interquartile range as descriptive statistics about pond areas. To compare the amphibian occurrence in ponds, we calculated the percentage of occupied ponds and we compared these percentages.

Incidence function model (IFM) is a spatially realistic model, which uses the formulas of Hanski's (1999) metapopulation model and the probability of pond occupancy can be predicted. Using this model, we determined those pond area thresholds for each amphibian species, where the probability of occupancy is higher than 0.5. This model type contributes to species-specific conservation measures which can be focused also on different pond area ranges. The IFMs were implemented in the R environment (R Core Team, 2021) using the tutorial of Oksanen (2004). In this implementation IFM is a special generalized linear model with binomial error distribution, which uses the natural logarithm of habitat patches' size as independent variable and the occupancy state of habitat patches (1 if occupied, else 0) as dependent variable. The model takes into consideration the isolation of habitat patches by adding isolation values to patch areas as linear predictors (see Oksanen, 2004). The isolation of a habitat patch is given by: $\sum exp\ (-dij \times\ \alpha) pjAj$, where *dij* is the distance between patch *i* and *j* patch, α is the inverse of dispersal rate of the species (Tab. 1), *pj* is the occupancy state and *Aj* is the area of *j* habitat patch. Using IFMs we extracted pond area values with predicted occupancy probability at least 0.5 for each amphibian species and we compared them using Kruskal-Wallis H-test and Pairwise Wilcoxon Test with Holm *p*-value adjustment.

The Stochastic Patch Occupancy Model (SPOM) is also a spatially realistic stochastic model using colonization and extinction probabilities which simulates the pond occupancy over time. The model starts from occupancy states in timestep *t* and determines how the patch occupancy (i.e., presence-absence of the species) changes from time *t* to time *t+1*. If a patch of habitat was not occupied at time t, but is close enough to other patches occupied at time t, it is likely to be occupied at time t+1 based on the colonisation and extinction probabilities. The model requires the specification of colonization and extinction functions, therefor we assumed that colonization and extinction can be described by the formulas of Hanski (1994, 1999) (see formulas summarized in *MetaLandSim* R package documentation; Mestre *et al.*, 2016). We assumed that the area and number of habitat patches do not vary over time. This assumption is used to answer our third objective, highlighting how patch occupancy would change over a 25-year time frame if the study area remains in its current state. SPOMs were run 1000 times and the average of the results was plotted for each species.

Both IFM and SPOM models are necessary to take into consideration the species-specific dispersal distances. For our species dispersal distances are summarized in Tab. 1 based on Trochet *et al.* (2014).

Table 1. Dispersion distance of amphibian species considered in this study.

Species	Dispersal distance (m)
Rana dalmatina	1500
Bombina variegata	1200
Pelophylax complex	800
Hyla arborea	2200
Lissotriton vulgaris ampelensis	1000
Triturus cristatus	1000

Results

Pond characteristics, occurrence and number of individuals

15 ponds were identified during the three sampling occasions in 2022, 12 of which were identified in 2019 too. Of the 19 ponds identified during the survey performed in 2019, 7 did not form in 2022 (Fig. 1). Taking into account that 5 out of 15 ponds found in 2022 were permanent-like ponds, the temporary pond loss was 78% compared to 2009 data (n = 47) reported in Erős *et al*. (2020). The pond area ($median_{2022}$ = 160 m^2, IQR_{2022}: 78 – 1218 m^2) has not

changed since the 2019 survey (median$_{2019}$ = 176 m^2, IQR$_{2019}$: 66 – 290 m^2; Mann-Whitney U-test: W = 121.5, p-value = 0.48). All amphibian species known from the area were found, except *Pelobates fuscus*.

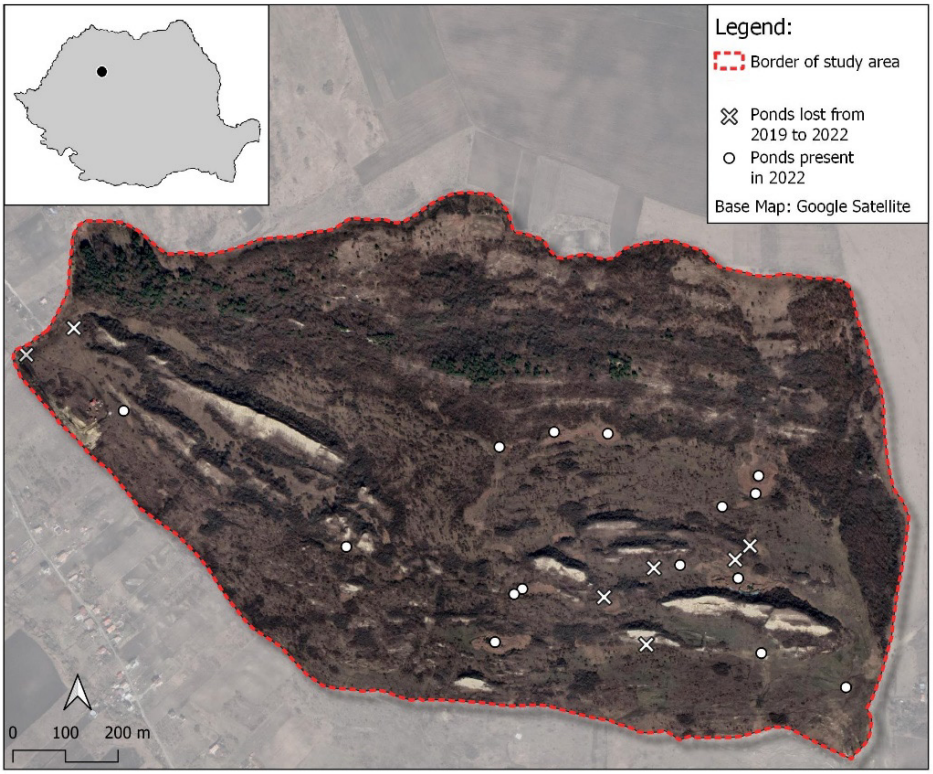

Figure 1. Map of study area. The black dot denotes the position of study area in Romania, while white dots denote ponds existing in 2022 and crosses denote ponds that existed in 2019 but dried out in 2022. The map was generated using Google Satellite.

In 2022, *Rana dalmatina* was found in the highest number of ponds, while *Hyla arborea* was found in the lowest number of ponds, and only during night-surveys (Fig. 2). Overall, day surveys supplemented with night surveys resulted in higher patch occupancy values for each species (Fig. 2). We found no difference in the total number of individuals between the day and night survey during the first (W = 33, p-value = 0.072; Fig. 2) and the last survey (W = 78, p-value = 0.144), but we found significantly higher number of individuals in the second day-survey (W = 114, p-value = 0.04; Fig. 2).

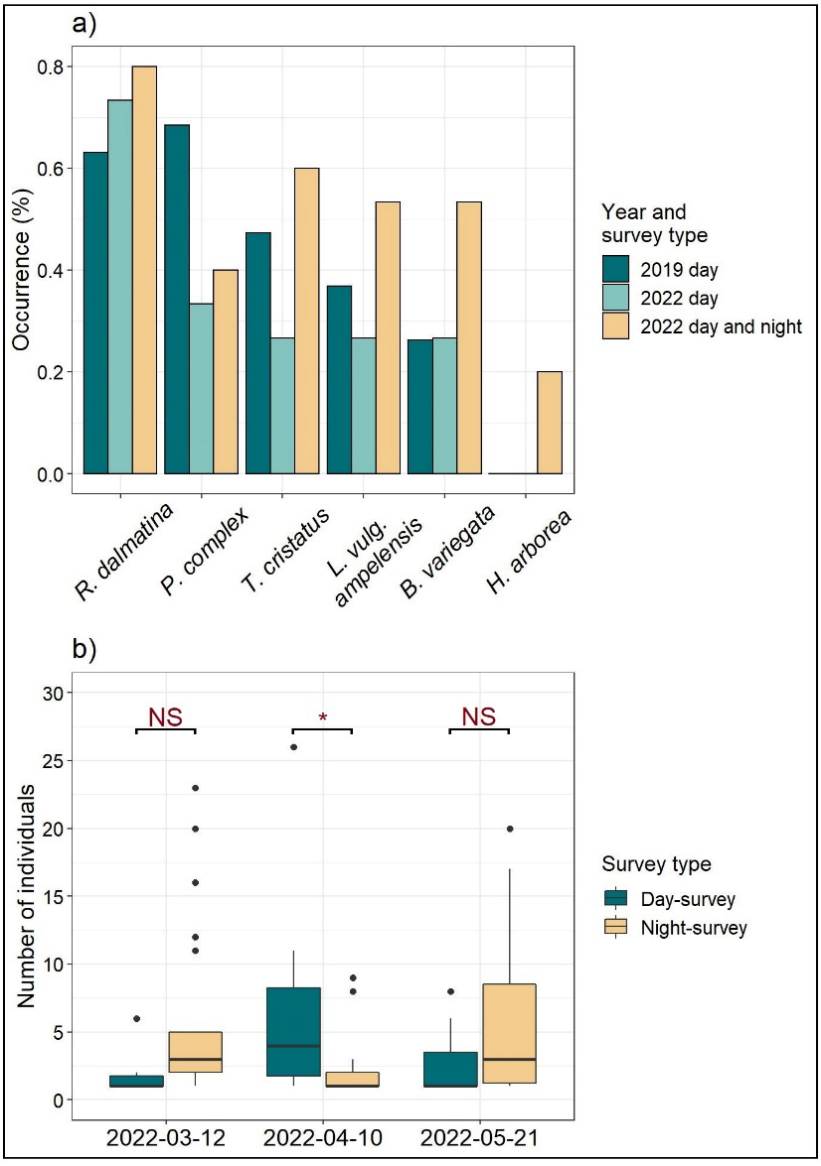

Figure 2. Panel a) amphibian species occurrence in ponds by survey year and type. We note that pond number in 2019 was 19, while in 2022 was 15; panel b) Cumulative umber of individuals in ponds by survey type, "NS" denotes non-significant differences between number of individuals by survey type, while "*" denotes p-value < 0.05 of Mann-Whitney U-test.

Incidence function models

Pond area was not found to be a significant factor of pond occupancy in the case of *Hyla arborea* and *Pelophylax* complex (Tab. 2). The model estimate of *Pelophylax* complex was unreliable, with lower estimate value than its standard error. Incidence function model estimates were similar in the case of *Triturus cristatus* and *Lissotriton vulgaris ampelensis* (Tab. 2).

Comparing pond area values by occupancy probability greater than 0.5, we found that *Rana dalmatina* and *Pelophylax* complex occupied significantly smaller ponds than other species (Kruskal-Wallis H-test: $\chi^2 = 29.8$, df = 4, p-value <0.001; Fig. 4). *Triturus cristatus*, *Lissotriton vulgaris ampelensis* and *Bombina variegata* occupied ponds with similar area (Fig. 3). The IFM model did not predict occupancy probability larger than 0.5 for *Hyla arborea*, therefore this species was excluded from the comparison and is not presented in Fig. 3.

Table 2. Incidence function model estimates: the effect of pond area on occupancy. [a]p-value is presented if it is significant; "NS" denotes non-significant p-value.

Species	Estimate	SE	z-value	p-value[a]
Rana dalmatina				
Intercept	-25.5	2.2	-11.6	<0.001
log(Pond area)	1.34	0.48	2.8	0.005
Pelophylax complex				
Intercept	-18.37	1.1	-16.68	<0.001
log(Pond area)	0.035	0.2	0.18	NS
Triturus cristatus				
Intercept	-21.76	1.25	-17.4	<0.001
log(Pond area)	0.5	0.22	2.23	0.023
Lissotriton vulgaris ampelensis				
Intercept	-21.66	1.28	-16.92	<0.001
log(Pond area)	0.49	0.22	2.21	0.03
Bombina variegata				
Intercept	-21.59	1.27	-16.98	<0.001
log(Pond area)	0.44	0.22	2	0.046
Hyla arborea				
Intercept	-23.48	3.3	-7.12	<0.001
log(Pond area)	0.73	0.49	1.5	NS

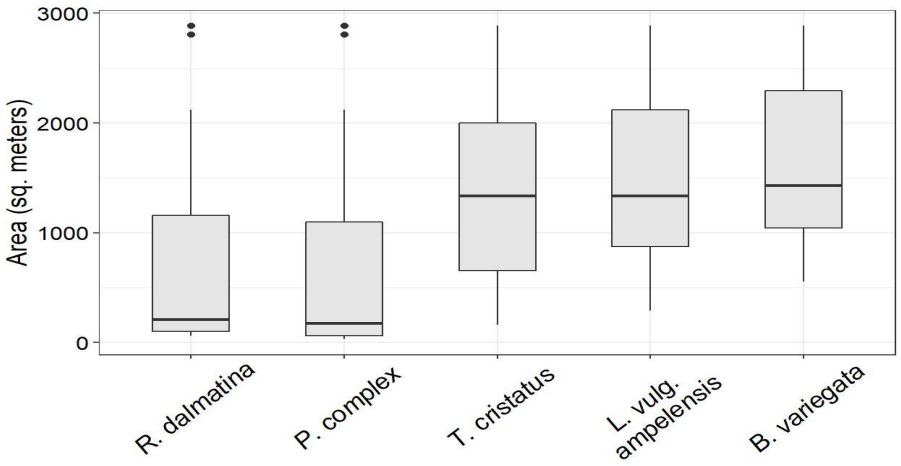

Figure 3. Pond area for species where predicted pond occupancy probability by incidence function models was greater than 0.5.

Stochastic patch occupancy models

Stochastic patch occupancy models showed that the patch occupancy of the species can increase over time, if the landscape will not change, i.e., the number of ponds and their area will not decrease (Fig. 4). During stochastic patch occupancy modelling, the highest patch occupancy was achieved by *Rana dalmatina*, while the lowest was achieved by *Triturus cristatus* and the two species belonging to the *Pelophylax* complex (Fig. 4).

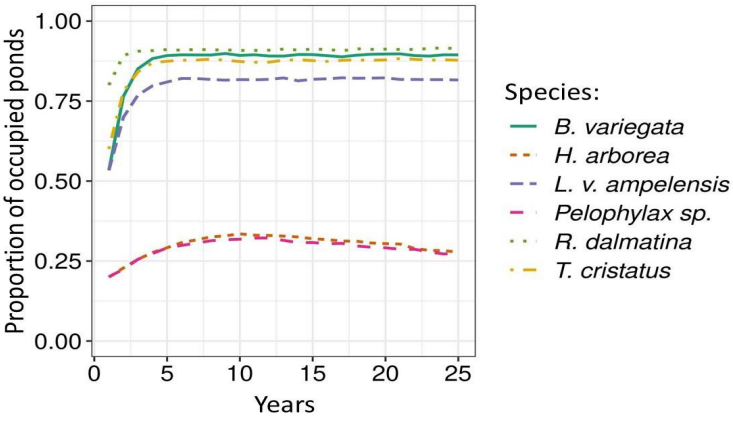

Figure 4. Results of stochastic patch occupancy models projected to a 25-year timeframe if the habitat patches and surrounding landscape remain in their current state.

Discussion

The data collected from a study area regarding the presence or abundance of target species depends largely on the survey method used to collect given data. Therefore, we surveyed our study area three times to get reliable data on pond occupancy and abundance of amphibian species inhabiting the study area. Twice in three surveys we found higher number of individuals at night surveys than during daytime. Combining day and night surveys is important, because the detectability of amphibian species may vary between years and species (see discussed in Schmidt, 2005), under different environmental variables (e.g., rainfall, temperature), even as the days of the year progress (Petitot *et al.*, 2014), and is highly dependent on the experience of the surveyor (Sewell *et al.*, 2010).

The incidence function modelling based on presence-absence data of species derived both from day and night survey showed that all ponds, regardless of size, are occupied by different amphibian species. *Triturus cristatus*, *Lissotrition vulgaris ampelensis* and *Bombina variegata* occupied with higher probability (larger than 0.5) ponds larger than 1000 m^2 (Fig. 3), while *Rana dalmatina* and species from *Pelophylax* complex occupied smaller ones. Unfortunately, the number of temporary ponds is in a constant decrease, i.e., less temporary ponds were formed in 2022 than in 2019, but the temporary pond loss comparing to 2009 data was 78% (REF). Therefore, conservation activities should address all pond types to ensure the persistence of all amphibian populations. Pond loss is recognized as a major threat to the amphibian population (Arntzen *et al.*, 2017; Cushman, 2006; Erős et al., 2020).

Successful creation and maintenance of ponds to conserve valuable aquatic habitats is a great challenge (e.g., Collserola Natural Park's project; Pinto-Cruz et al., 2017). The well-maintained ponds with longer hydroperiods have higher biodiversity in traditional landscapes (Hartel et al., 2014) and in urban environments as well (Beja and Alcazar, 2003; Oertli and Parris, 2019). In our study area, the increase in shrub cover over the last decade (Erős *et al.,* 2020) may be a major threat to pond formation and sustainability. The curbing of this vegetation growth could be an initial step in conserving ponds as in the case of *Epidalea calamita* frog (Buckley *et al.,* 2014; McGrath and Lorenzen, 2010). Another successfully deployed active conservation measure is the implementation of water pumps to actively maintain the hydroperiodicity of ponds (Mathwin *et al.*, 2021).

Based on our SPOMs, the under a constant landscape and compared to other species inhabiting our study area, the patch occupancy of the *Rana dalmatina* could be the highest of the studied species in the next 25 years. Our

model accounts for an ideal state; however, long-term studies performed on this species have shown that population size fluctuations are influenced by stochastic conditions (e.g., weather) and population density (Hartel and Öllerer, 2009) or the presence of predators (Schmidt *et al.,* 2021). Indeed, small populations may be more susceptible to extinction due to stochastic events than larger populations (Pellet and Schmidt, 2005). The ideal state represented by our SPOM can be affected in several ways (e.g., changes in climate and habitat parameters, or in biotic interactions). Climate change has already resulted in the inclusion of several amphibian species from Australia on the IUCN Red List (Hero *et al.*, 2006), and might be a contributing factor to the loss of amphibian biodiversity in Europe as well (Popescu *et al.*, 2013). However, there are surprisingly few examples of specific climate change measures being integrated into regional or local amphibian management practices (Shoo *et al.*, 2011). Changes in habitat parameters (e.g., hydroperiodicity, temperature) or competition between amphibian species have negative impacts on the survival of species, and need to be accounted for during protected area management (Cayuela *et al.*, 2018; Roth *et al.*, 2016; Tournier *et al.*, 2017).

Conclusions

Day and night surveys can complement each other to provide a good quality snapshot of the amphibian species and their patch occupancy. In this study we show that the number of ponds, mostly the number of temporary ponds, decreases over time. Local populations of amphibian species can be conserved if there will be no significant negative changes in habitat and landscape, or if protective measures will be implemented. The continuation of small-scale monitoring activities and awareness raising regarding the disappearance of ponds are essential to protect and conserve the remaining local populations of amphibians in the protected area.

Data availability. All data and scripts are available by request by contacting the corresponding author via e-mail.

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References

Akeroyd, J. R., & Page, N. (2007). The Saxon Villages of Southern Transylvania: Conserving Biodiversity in a Historic Landscape. In *Nature Conservation* (pp. 199–210). Berlin, Heidelberg: Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-540-47229-2_21

Arntzen, J. W., Abrahams, C., Meilink, W. R. M., Iosif, R., & Zuiderwijk, A. (2017). Amphibian decline, pond loss and reduced population connectivity under agricultural intensification over a 38-year period. *Biodiversity and Conservation*, *26*(6), 1411–1430. https://doi.org/10.1007/s10531-017-1307-y

Beja, P., & Alcazar, R. (2003). Conservation of Mediterranean temporary ponds under agricultural intensification: an evaluation using amphibians. *Biological Conservation*, *114*(3), 317–326. https://doi.org/10.1016/S0006-3207(03)00051-X

Blaustein, A. R., & Kiesecker, J. M. (2002). Complexity in conservation: lessons from the global decline of amphibian populations. *Ecology Letters*, *5*(4), 597–608. https://doi.org/10.1046/j.1461-0248.2002.00352.x

Bolochio, B. E., Lescano, J. N., Cordier, J. M., Loyola, R., & Nori, J. (2020). A functional perspective for global amphibian conservation. *Biological Conservation*, *245*(March), 108572. https://doi.org/10.1016/j.biocon.2020.108572

Buckley, J., Beebee, T. J. C., & Schmidt, B. R. (2014). Monitoring amphibian declines: population trends of an endangered species over 20 years in Britain. *Animal Conservation*, *17*(1), 27–34. https://doi.org/10.1111/acv.12052

Calhoun, A. J. K., Mushet, D. M., Bell, K. P., Boix, D., Fitzsimons, J. A., & Isselin-Nondedeu, F. (2017). Temporary wetlands: challenges and solutions to conserving a "disappearing" ecosystem. *Biological Conservation*, *211*, 3–11. https://doi.org/10.1016/j.biocon.2016.11.024

Cayuela, H., Besnard, A., Quay, L., Helder, R., Léna, J.-P., Joly, P., & Pichenot, J. (2018). Demographic response to patch destruction in a spatially structured amphibian population. *Journal of Applied Ecology*, *55*(5), 2204–2215. https://doi.org/https://doi.org/10.1111/1365-2664.13198

Cogălniceanu, D., Băncilă, R., Plăiaşu, R., Samoilă, C., & Hartel, T. (2012). Aquatic habitat use by amphibians with specific reference to *Rana temporaria* at high elevations (Retezat Mountains National Park, Romania). *Annales de Limnologie - International Journal of Limnology*, *48*(4), 355–362. https://doi.org/10.1051/limn/2012026

Collserola Natural Park's project: https://www.europarc.org/case-studies/pond-management-conservation-amphibians-collserola-natural-park/

Curado, N., Hartel, T., & Arntzen, J. W. (2011). Amphibian pond loss as a function of landscape change - A case study over three decades in an agricultural area of northern France. *Biological Conservation*, *144*(5), 1610–1618. https://doi.org/10.1016/j.biocon.2011.02.011

Cushman, S. A. (2006). Effects of habitat loss and fragmentation on amphibians: A review and prospectus. *Biological Conservation*, *128*(2), 231–240. https://doi.org/10.1016/j.biocon.2005.09.031

Erős, N., Maloș, C. V., Horváth, C., & Hartel, T. (2020). Temporary pond loss as a result of pasture abandonment: exploring the social-ecological drivers and consequences for amphibians. *Journal for Nature Conservation*, *55*(October 2019), 125836. https://doi.org/10.1016/j.jnc.2020.125836

Grodzińska-Jurczak, M., & Cent, J. (2011). Can public participation increase nature conservation effectiveness? *Innovation: The European Journal of Social Science Research*, *24*(3), 371–378. https://doi.org/10.1080/13511610.2011.592069

Hanski, I. (1994). A practical model of metapopulation dynamics. *Journal of Animal Ecology*, 151–162.

Hanski, I., & others. (1999). *Metapopulation ecology*. Oxford University Press.

Hartel, T., & Öllerer, K. (2009). Local turnover and factors influencing the persistence of amphibians in permanent ponds from the saxon landscapes of transylvania. *North-Western Journal of Zoology*, *5*(1), 40–52.

Hartel, T., & von Wehrden, H. (2013). Farmed areas predict the distribution of amphibian ponds in a traditional rural landscape. *PLoS ONE*, *8*(5), 1–8. https://doi.org/10.1371/journal.pone.0063649

Hartel, T., Sos, T., Popescu, V. D., Băncilă, R. I., Cogălniceanu, D., & Rozylowicz, L. (2014). Amphibian conservation in traditional cultural landscapes: the case of Central Romania. *North-Western Journal of Zoology*, *10*(Supplement 1), S51-S61.

He, S., Yang, L., & Min, Q. (2020). Community Participation in Nature Conservation: The Chinese Experience and Its Implication to National Park Management. *Sustainability*, *12*(11), 4760. https://doi.org/10.3390/su12114760

Hero, J.-M., Morrison, C., Gillespie, G., Dale Roberts, J., Newell, D., Meyer, E., … Shoo, L. (2006). Overview of the conservation status of Australian frogs. *Pacific Conservation Biology*, *12*(4), 313. https://doi.org/10.1071/PC060313

Ives, C. D., Abson, D. J., von Wehrden, H., Dorninger, C., Klaniecki, K., & Fischer, J. (2018). Reconnecting with nature for sustainability. *Sustainability Science*, *13*(5), 1389–1397. https://doi.org/10.1007/s11625-018-0542-9

MacKenzie, D. I. (2005). What are the issues with presence-absence data for wildlife managers? *Wildlife Research*, *69*(3), 849–860. https://doi.org/10.2193/0022-541X(2005)069

MacKenzie, D. I., Nichols, J. D., Lachman, G. B., Droege, S., Royle, A. A., & Langtimm, C. A. (2002). Estimating site occupancy rates when detection probabilities are less than one. *Ecology*, *83*(8), 2248–2255. https://doi.org/10.1890/0012-9658(2002)083[2248:ESORWD]2.0.CO;2

Mathwin, R., Wassens, S., Young, J., Ye, Q., & Bradshaw, C. J. A. (2021). Manipulating water for amphibian conservation. *Conservation Biology*, *35*(1), 24–34. https://doi.org/10.1111/cobi.13501

McGrath, A. L., & Lorenzen, K. (2010). Management history and climate as key factors driving natterjack toad population trends in Britain. *Animal Conservation*, *13*(5), 483–494. https://doi.org/10.1111/j.1469-1795.2010.00367.x

Mestre, F., Canovas, F., Pita, R., Mira, A., & Beja, P. (2016). An R Package for simulating metapopulation dynamics and range expansion under environmental change. *Environmental Modelling and Software*.

Nori, J., Lemes, P., Urbina-Cardona, N., Baldo, D., Lescano, J., & Loyola, R. (2015). Amphibian conservation, land-use changes and protected areas: A global overview. *Biological Conservation*, *191*(October 2017), 367–374. https://doi.org/10.1016/j.biocon.2015.07.028

Oertli, B., & Parris, K. M. (2019). Review: Toward management of urban ponds for freshwater biodiversity. *Ecosphere*, *10*(7). https://doi.org/10.1002/ecs2.2810

Oksanen, J. (2004). *Incidence Function Model in R*. https://www.researchgate.net/publication/239921870_Incidence_Function_Model_in_R

Pellet, J., & Schmidt, B. R. (2005). Monitoring distributions using call surveys: estimating site occupancy, detection probabilities and inferring absence. *Biological Conservation*, *123*(1), 27–35. https://doi.org/10.1016/j.biocon.2004.10.005

Petitot, M., Manceau, N., Geniez, P., & Besnard, A. (2014). Optimizing occupancy surveys by maximizing detection probability: application to amphibian monitoring in the Mediterranean region. *Ecology and Evolution*, *4*(18), 3538–3549. https://doi.org/10.1002/ece3.1207

Pinto-Cruz, C., Marques, J., Lumbreras, A., Meireles, C., Belo, A., Sousa, L., … Lúcio, C. (2017). Ecological restoration of Mediterranean temporary ponds in Portugal. *7th European Pond Conservation Network Workshop + LIFE Charcos Seminar*, 50.

Popescu, V. D., Rozylowicz, L., Cogălniceanu, D., Niculae, I. M., & Cucu, A. L. (2013). Moving into Protected Areas? Setting Conservation Priorities for Romanian Reptiles and Amphibians at Risk from Climate Change. *PLoS ONE*, *8*(11), e79330. https://doi.org/10.1371/journal.pone.0079330

R Core Team. (2021). *R: A Language and Environment for Statistical Computing*. Vienna, Austria. Retrieved from https://www.r-project.org/

Robinson, J. G. (2006). Conservation Biology and Real-World Conservation. *Conservation Biology*, *20*(3), 658–669. https://doi.org/10.1111/j.1523-1739.2006.00469.x

Roth, T., Bühler, C., & Amrhein, V. (2016). Estimating effects of species interactions on populations of endangered species. *The American Naturalist*, *187*, 457–467. https://doi.org/https://doi.org/10.1086/685095

Schmidt, B. R., Băncilă, R. I., Hartel, T., Grossenbacher, K., and Schaub, M. (2021). Shifts in amphibian population dynamics in response to a change in the predator community. *Ecosphere*, *12*(5), e03528. https://doi.org/10.1002/ecs2.3528

Schmidt, B.R. (2005). Monitoring the distribution of pond-breeding amphibians when species are detected imperfectly. *Aquatic Conservation: Marine and Freshwater Ecosystems*, *15*, 681-692. https://doi.org/10.1002/aqc.740

Sewell, D., Beebee, T. J. C., & Griffiths, R. A. (2010). Optimising biodiversity assessments by volunteers: The application of occupancy modelling to large-scale amphibian surveys. *Biological Conservation*, *143*(9), 2102–2110. https://doi.org/10.1016/j.biocon.2010.05.019

Shoo, L. P., Olson, D. H., McMenamin, S. K., Murray, K. A., Van Sluys, M., Donnelly, M. A., … Hero, J.-M. (2011). Engineering a future for amphibians under climate change. *Journal of Applied Ecology*, *48*(2), 487–492. https://doi.org/10.1111/j.1365-2664.2010.01942.x

Sos, T., & Hegyeli, Z. (2015). Characteristic morphotype distribution predicts the extended range of the "Transylvanian" smooth newt, *Lissotriton vulgaris ampelensis* Fuhn 1951, in Romania. *North-Western Journal of Zoology*, *11*(1), 34–40.

Strohbach, M. W., Kohler, M. L., Dauber, J., & Klimek, S. (2015). High Nature Value farming: From indication to conservation. *Ecological Indicators*, *57*(2015), 557–563. https://doi.org/10.1016/j.ecolind.2015.05.021

Tournier, E., Besnard, A., Tournier, V., & Cayuela, H. (2017). Manipulating waterbody hydroperiod affects movement behaviour and occupancy dynamics in an amphibian. *Freshwater Biology*, *62*, 1768– 1782. https://doi.org/https://doi.org/10.1111/fwb.12988

Trochet, A., Moulherat, S., Calvez, O., Stevens, V. M., Clobert, J., & Schmeller, D. S. (2014). A database of life-history traits of European amphibians. *Biodiversity Data Journal*, *2*, e4123. https://doi.org/10.3897/BDJ.2.e4123

Walls, S. C., & Gabor, C. R. (2019). Integrating Behavior and Physiology Into Strategies for Amphibian Conservation. *Frontiers in Ecology and Evolution*, *7*(JUN), 1–13. https://doi.org/10.3389/fevo.2019.00234