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A case report and a review
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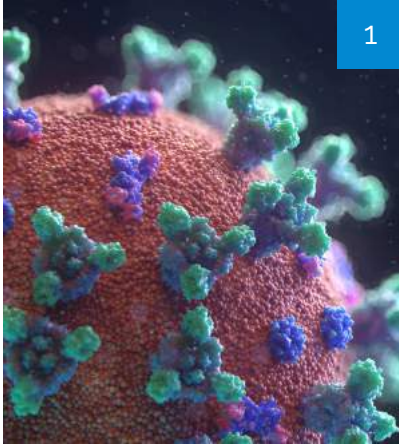
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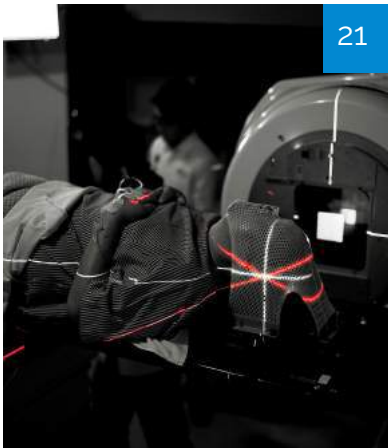
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Gastrointestinal Bleeding in Patients with Severe COVID 19: A Case Report and a Review of the Literature

*Mariana A. Zanni MD, Giuliana Ponton MD, Fabiana Durante MD, Sergio Sitta MD MAAC
& Nathaniel Dionisio MD MAAC*

BACKGROUND

SARS-CoV-2 (Severe acute respiratory syndrome coronavirus 2) is a positive, encapsulated and non-segmented RNA virus that belongs to the Coronavirus family [1].

In December 2019, an outbreak of pneumonia of unknown cause was reported in Wuhan, Hubei Province, China. This new betacoronavirus became a threat to public health and sustained mandatory social and preventive isolation as a containment measure.

Argentina was one of the few countries that adopted sustained mandatory social and preventive isolation as a containment measure. However, it is among the 10 countries with the most infections in the world. The National Health Ministry reported 1,090,589 confirmed cases to date, with 28,896 deaths [4]. The mortality rate of the virus in our country is 4.7%.

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Gastrointestinal Bleeding in Patients with Severe COVID 19: A Case Report and a Review of the Literature

Mariana A. Zanni MD^a, Giuliana Ponton MD^o, Fabiana Durante MD^p, Sergio Sitta MD MAAC^o
& Nathaniel Dionisio MD MAAC^{*}

Department of General Surgery, Sanatorio Municipal "Dr. Julio Mendez", CABA, Argentina.

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However, it is among the 10 countries with the most infections in the world. The National Health Ministry reported 1,090,589 confirmed cases to date, with 28,896 deaths [4]. The mortality rate of the virus in our country is 4.7%.

There are multiple forms of presentation of the disease. Among them, respiratory and systemic symptoms are the most frequent. Between 3% and 50% of patients present with gastrointestinal symptoms including nausea, vomiting, abdominal pain, and diarrhea [5].

COVID-19 damages the digestive system both directly and via inflammatory response and indirectly by viral invasion. COVID 19 has been shown to use Angiotensin-Converting enzyme II (ACE II) receptors for entry into the cell. ACE II is highly expressed in type II alveolar cells of the lung and epithelial cells of the gastrointestinal system [6].

Gastrointestinal system bleeding is rare and is frequently seen in critical intensive care patients [7][8]. In some cases, and due to the severe inflammatory stage, it is associated with a procoagulant state caused by endothelial damage, with elevation of D-dimer, fibrinogen, thrombopenia, with the consequent excessive production of platelets, triggering as a consequence arterial and venous thrombotic events and disseminated intravascular coagulation (DIC), leading to increasingly frequent severe bleeding events. [16]

We have carried out a review of the literature on gastrointestinal bleeding in patients affected by COVID 19. In this article we will present the conclusions of this review and present the case of a patient hospitalized for severe SARS-CoV-2 associated with gastrointestinal bleeding.

I. CASE REPORT

A 45-year-old male patient presented in the emergency department with fever and progressive dyspnea. He had a medical history of pulmonary tuberculosis, smoking, obesity (BMI 37). The vital signs were blood pressure: 140/90 mmHg, pulse: 103/ beats.min, O₂ saturation 99%, respiration rate 17, Glasgow Coma Score: 15 (G4M6S5). The value of D dimer at admission was 787,5 ng/ml.

At admission a nasopharyngeal swab was performed which tested positive for SARS-CoV-2 infection and chest CT scan showed bilateral interstitial pneumonia (Figure 1).

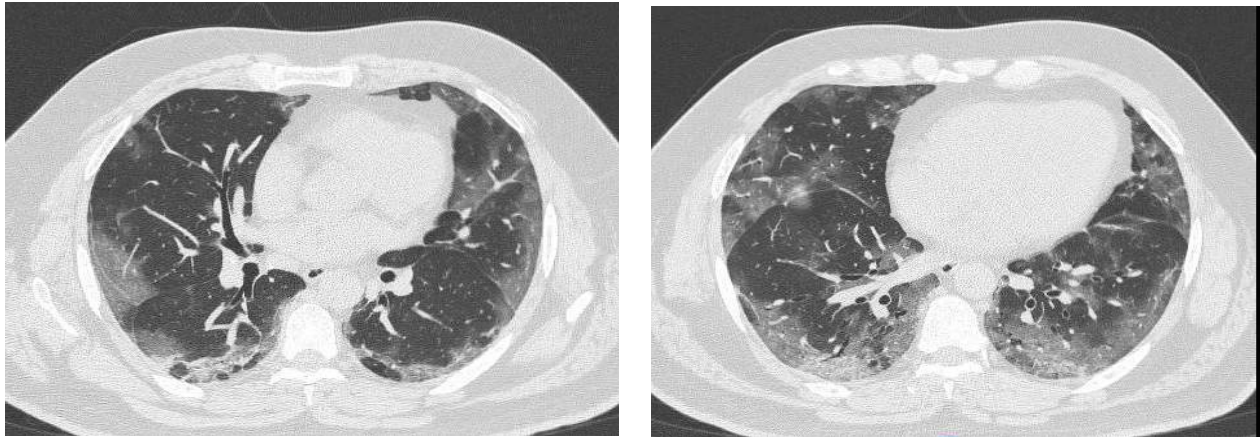


Figure 1: Chest CT Scan

He evolved with a progression of his dyspnea, fever and desaturation that didn't reverse with high-flow oxygen with a 100% reservoir mask. He was transferred to the intensive care unit, with a diagnosis of severe respiratory failure, orotracheal intubation was performed, with the requirement of prone cycles and antibiotic therapy.

During his hospitalization in the intensive care unit he presented multiple complications, and the need for a variety of advanced vital supports, for example: adrenal insufficiency, septic shock with multiple bacteriological rescues and antibiotic therapy, acute renal failure requiring hemodialysis, liver failure, myoclonus in the right

hemibody, prolonged assisted ventilation and tracheostomy, severe malnutrition, alithiasic cholecystitis that required percutaneous cholecystostomy and disseminated intravascular coagulation.

During his evolution, he presented two episodes of lower gastrointestinal bleeding, colonoscopy didn't reveal any tumor or sites of active bleeding. In his third episode, he was treated with support measures and angiography, with no evidence of active bleeding, so after failure of conservative treatment, a right colectomy and diverting loop ileostomy was performed.



Figure 2: A total opening of the surgical specimen was observed in a sector of 9 x 3.5 cm mucosa of blackish coloration, with multiple depressions of rough surface, ulcerated appearance. Rest of the mucosa with preservation of folds, of edematous appearance. The ileum exhibits mucosa with

preservation of the folds. From the adipose tissue of the meso, 4 lymph nodes are resected, the largest of 0.6 x 0.3 cm, when cutting homogeneous brownish tissue.

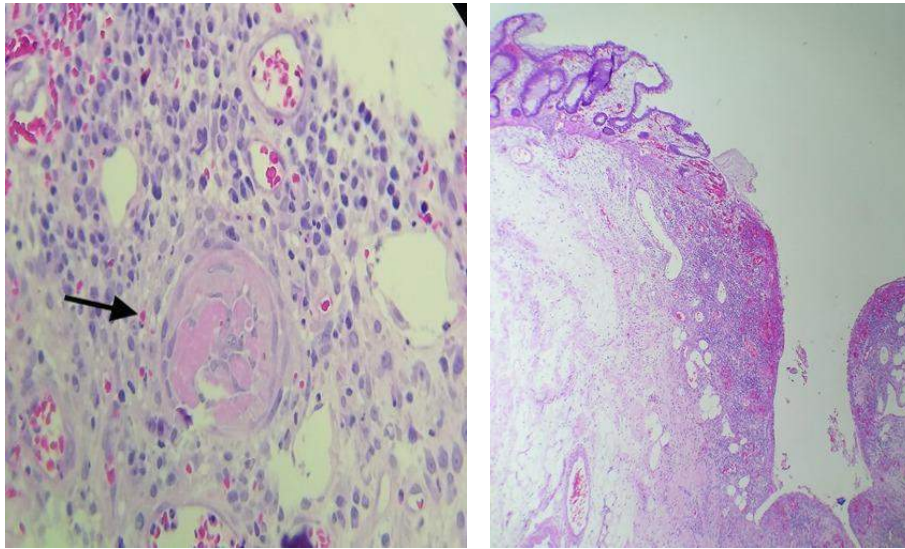


Figure 3 and 4: The different histological sections showed a colon wall with multiple areas with extensive ulceration that involves even the submucosa, with a dense lymphoplasmacytic infiltrate with areas of erythrocyte extravasation. In the lamina propria, small-caliber vessels with fibrinoid thrombi that completely occlude the lumen are observed (black arrow). In the submucosa there is intense edema and in some areas atrophy of the muscular tunica propria. The inflammatory process described above spreads diffusely.

However, he later presented another episode of gastrointestinal bleeding with porraceous discharge from the nasogastric tube, an endoscopy was performed that shows: "congestive-erosive gastropathy, without bleeding stigmata".

After this last episode, the patient evolved favorably, hospital discharge was granted fifty days later.

II. DISCUSSION

We present a case of covid-19 disease with an atypical presentation in the form of severe gastrointestinal bleeding associated with disseminated intravascular coagulation. This is another case that suggests that SARS-CoV-2 can trigger a severe thrombotic microangiopathy which can end up with uncontrolled bleeding, due to endothelial lung damage resulting in microthrombi formation and platelet consumption. The pathogenesis of thrombocytopenia could also recognise other mechanisms [9] [10]:

- Development of autoantibodies or immune complexes.
- Direct infection of hematopoietic stem / progenitor cells: following virus infection, the cytokine storm destroys bone marrow cells and leads to a decrease in platelet production.

During hospitalization in the intensive care unit for severe COVID 19 with respiratory failure, he presented three episodes of gastrointestinal bleeding with hemodynamic decompensation, requiring vasoactive drugs and transfusion of blood products. Four endoscopies were performed which did not achieve hemostatic treatment and two negative angiographies, hindering the algorithm and delaying surgical treatment. Guidelines advise that patients who present with acute upper GI bleeding should undergo endoscopy within 24 hours from presentation [11]. However, the discussion for endoscopy in patients with COVID 19 pneumonia brings about unique management decisions. Although endoscopy can provide a solution if a discrete

visible vessel is seen, the risk of the procedure may outweigh the benefit in patients with COVID 19 pneumonia. In the study of Kimberly Cavaliere et al they decided to treat these patients conservatively with a proton pump inhibitor drip, blood transfusion as needed, and frequent monitoring of vital signs, GI symptoms, and hemoglobin value. Endoscopy was reserved if the patient did not respond to conservative management by 24 hours (lack of hemodynamic stability and if the hemoglobin was not stable). Cessation of clinical symptoms of acute upper GI bleeding was seen in all of their patients in combination with stabilization of hemoglobin. None of the patients required upper endoscopy during their clinical course [12].

It has recently been shown that delaying the endoscopy for 24 hours has not affected 30-day mortality in comparison with earlier endoscopy. [13]

According to the current literature, the procoagulant state associated with SARS-CoV-2 is due to a systemic inflammatory response, producing high levels of D-dimer (direct relationship with increased mortality) and in more severe cases DIC with prolongation of PT and KTTTP . Therefore, it has been shown to be predictive of severity and mortality, which could require more aggressive treatment due to the increased risk of severe bleeding with a difficult endoscopic management. Likewise, serial coagulation tests should be performed in the initial stages of the disease, seeking early detection of coagulopathy for intensive clinical management. Given the severity of the patient's condition and the strong association of SARS-CoV 2 infection with DIC, it should be sought and treated intensively [14].

III. CONCLUSION

In conclusion, digestive bleeding in patients with COVID 19 should be managed conservatively. The endoscopy should be performed for those who failed with conservative treatment. We propose this algorithm for those patients with gastrointestinal bleeding and severe COVID 19 Figure 6.

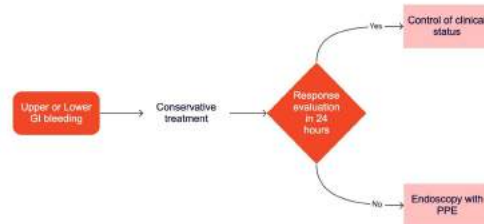


Figure 6: PPE (Personal protective equipment)

Declaration of Competing Interest

None

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None

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Oral Health Promotion in Children with Microcephaly After Social Isolation by COVID 19: Experience Report

*Dara Vitória Pereira Lopes Silva, Felipe Barros Castro, Fernanda de Carvalho Reis, Mayana Narde Souza,
Rafael Almeida Monteiro, Taylline das Mercês Gonçalves, Francisco Xavier Paranhos Coelho Simões
& Maria da Conceição Andrade de Freitas*

ABSTRACT

The purpose of this study was to report extracurricular activities performed by professors and students of the Dentistry course at the State University of Southwest Bahia. Their goal was to promote oral health to microcephaly patients and their parents, after the period of social isolation due to the COVID-19 pandemic. In this context, a university extracurricular activity, more than ever, must assume its social responsibility, establishing new paths for public health care and regarding these actions correlating the academic community and society.

Keywords: microcephaly; public health; dentistry ; universities.

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Oral Health Promotion in Children with Microcephaly After Social Isolation by COVID 19: Experience Report

Dara Vitória Pereira Lopes Silva^a, Felipe Barros Castro^o, Fernanda de Carvalho Reis^o, Mayana Narde Souza^o, Rafael Almeida Monteiro^z, Taylline das Mercês Gonçalves^s, Francisco Xavier Paranhos Coelho Simões^x & Maria da Conceição Andrade de Freitas^v

ABSTRACT

The purpose of this study was to report extracurricular activities performed by professors and students of the Dentistry course at the State University of Southwest Bahia. Their goal was to promote oral health to microcephaly patients and their parents, after the period of social isolation due to the COVID-19 pandemic. In this context, a university extracurricular activity, more than ever, must assume its social responsibility, establishing new paths for public health care and regarding these actions correlating the academic community and society.

Keywords: microcephaly; public health; dentistry; universities.

I. INTRODUCTION

Throughout the history of undergraduate studies in Brazil, extracurricular activities were characterized by their assistance to local communities, often conducted towards low income citizens. However, it is commonly mistaken to assume that service is a one-way street. Extracurricular activities provide a dialogue between the university and society, where both environments teach and learn from each other, making the university not the only place where knowledge is shared. Consequently, professors and students must understand that in order to achieve a high quality education, the implementation of extracurriculars in their academic curriculum is extremely necessary. The Ministry of Education in Brazil included these activities in university courses through resolution number 7 in 2018. The National Education Plan was implemented for better qualification of future

professionals. This legal provision establishes that extracurricular activities - EA must comprise at least 10% of the total student curricular workload of undergraduate courses for the composition of curriculum.

During the period of social isolation due to high transmissibility of the new Coronavirus, universities had the relevant mission to supply technological, educational, cultural advances and community innovation with a role of social responsibility for their EA. In Brazil, universities' extracurricular practices were encouraged by promoting an educational, critical and ethical process through the implementation of Information and Communication Technologies in order to provide services in situations where it was necessary to overcome geographic, temporal, social and cultural barriers. In the scientific literature, an integrative review highlights studies that emphasize the use of teledentistry as a global reality, being an important tool to aid dental care, especially for the most vulnerable populations (TEIXEIRA et al, 2018), such as children diagnosed with microcephaly.

Currently, there is a lack of oral health education programs aimed at children with microcephaly, including a protocol for preventive dental care. Studies show parents' reports about the complexity of oral hygiene habits in their children's routine. Added to these factors, diet and continuous use of medications increase the incidence of oral pathologies. Additionally, there is an experience report of oral health promotion activities developed by professors and students of the Dentistry course to children with microcephaly after the social isolation resulting

from the COVID-19 health crisis, based on the principles of universality, equity and integrality.

II. METHODS

This is an observational study with a qualitative descriptive approach of a report of experience by undergraduate students and professors of the Dentistry course at the State University of Southwest Bahia (UESB). Since april 2017, the project named "Alvaro Marques Dental Center-Microcephaly" registered in UESB attendance services has been developed to promote oral health and dental care for individuals with microcephaly born in Jequié or nearby cities of Bahia, Brazil.

In COVID-19 pandemic period, oral health promotion activities were created by the academic team in a virtual format for individuals with microcephaly through their parents. The benefited public included 26 children diagnosed with microcephaly and/or congenital anomalies and syndromes registered in the project. Undergraduate students of the UESB Dentistry course under the guidance of professors, produced educational cards and videos about oral hygiene and dental development from July 2020 to November 2021. These materials were sent weekly to families through WhatsApp. There was also the participation of professors and students of the Nursing course, in 2021, with production of virtual cards/videos of health care for preventing COVID-19 contamination. They also promoted a virtual meeting with microcephaly children's parents, approaching mental health.

Nowadays, with the flexibility of physical and social isolation, it was possible, in October 2021, to return to dental practices in the Dentistry Module at UESB. These clinical activities are composed by collaborating professors of various Dentistry specialties such as Pediatric Dentistry, Periodontics, Maxillofacial Surgery and Endodontics. These professors are responsible for supervising the students according to the dental treatment plan and necessity of children with microcephaly. Also, educational cards and/or videos on oral hygiene were sent online once a week, and an academic team was available to

answer possible doubts and additional information by Whatsapp.

III. EXPERIENCE REPORT

University extracurricular activities concept emerges when the processes of public education begin to be discussed, at which time professors and students dialogue about the role of the university in social problems and inequalities. In the conception and practice of extracurricular activities guidelines in graduation, the articulation between teaching/research/EA is fundamental, anchored in interdisciplinary, educational, cultural, scientific and technological pedagogical processes. In 2021, this program had continuous activities, developed by the academic team linked to State University of Southwest Bahia (UESB), promoting oral health of children with microcephaly and their families.

In this project, extracurricular activities started in July 2020, in COVID-19 pandemic, offering online services of assistance and monitoring education in oral health. EA planned by the team consisted of guidance on topics such as cleaning teeth and tongue, gingival health and dental development through digital material, producing cards and videos for individuals with microcephaly and their respective families (Figure 1A and B). The chronology of tooth eruption caused the greatest digital interaction due to the constant participation of parents with children in the beginning of mixed dentition. The discussions generated by their questions provided a playful and scientific follow-up for everyone involved (Figure 2 A-D). The academic team realized the importance of online scientific activities on oral health in a more accessible language for this vulnerable population.



Figure 1: Video made by the project's students in a playful way about gingival health (A). Dental development card (B)

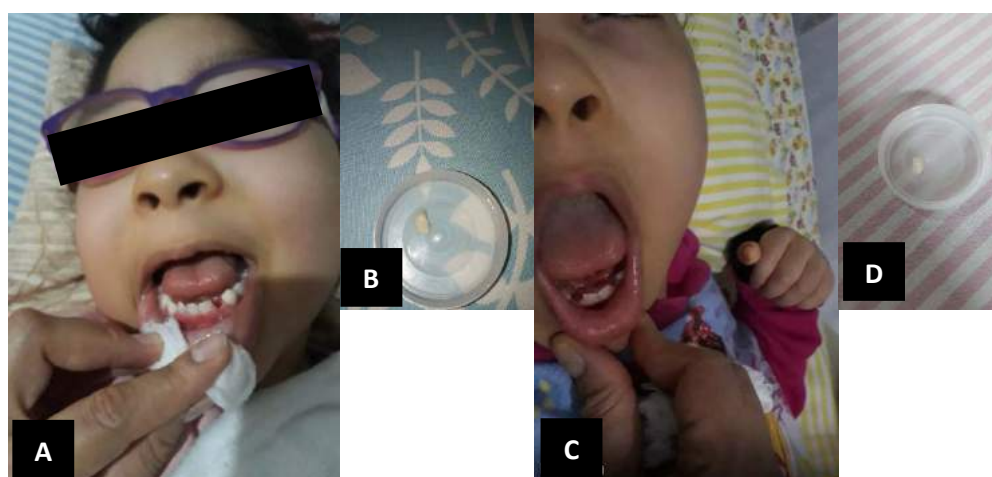


Figure 2: Digital resources provided by the mother of a child with microcephaly at the beginning of mixed dentition. Photograph shows her daughter's deciduous teeth loss (A-D)

One of the goals to obtain a differentiated academic formation is the humanized practice in public health services. In this context, after social isolation caused by COVID-19, clinical practices of dental care started assisting five children between five to eight years old with severe microcephaly, neuro psychomotor impairment, hearing and visual alterations. There were reports of medical stories after the pandemic period such as when one patient was diagnosed with Rett Syndrome and another with type I diabetes. A mother also reported that her child, with malnutrition, would have to use a naso-gastric tube. Parents of these patients related that they were using a pasty diet only during the day. There was also an increase in teeth grinding in the pandemic's isolation period. Concerning routine oral hygiene, three children

had their teeth brushed once a day without tongue brushing. In two children who had their teeth brushed three times a day, the clinical management was easier. To enable dental examination and clinical procedures, these conditioning techniques were used: voice control, musical distraction, protective stabilization of neck, hands and feet performed by the parent and students. On intraoral examination, children were beginning the mixed dentition. Excessive salivation, atypical anterior lingual interposition and normal tooth development were observed. Two children were diagnosed with drug-induced fibrous gingival hyperplasia that causes tooth eruption anomalies. They were indicated for surgery planning. All patients and their parents,

received a toothbrush and guidance about oral hygiene (Figure 3).



Figure 3: Clinical practice performed without physical stabilization on the patient with microcephaly in the mother's lap

The World Health Organization, an international reference on craniofacial anomalies, published a document entitled Global Strategies to reduce the Health-Care Burden of Craniofacial Anomalies. This document contained care protocol guidelines for this population, encompassing the need for oral health care for these patients (WHO, 2002). Children with microcephaly, in addition to the congenital anomaly due to the significant reduction in the occipitofrontal length when compared to those of the same sex and age, may present neuropsychomotor impairment (WHO, 2018). Studies report that these children are at greater risk of developing caries and periodontal

disease (MARINHO et al, 2020). In the present deciduous molars. The treatment consisted of mechanical removal of dental calculus with topical application of 2% sodium fluoride (Figure 4 A-C). According to parents' reports, there was great anxiety for improving their children's oral health. Full attendance to clinical practice was a reality in all families, despite the financial and urban mobility difficulties. The student's perception about extracurricular activities trajectories follows reflection of Pietrovski et al. (2018), which evidences interaction in professional and social scope.

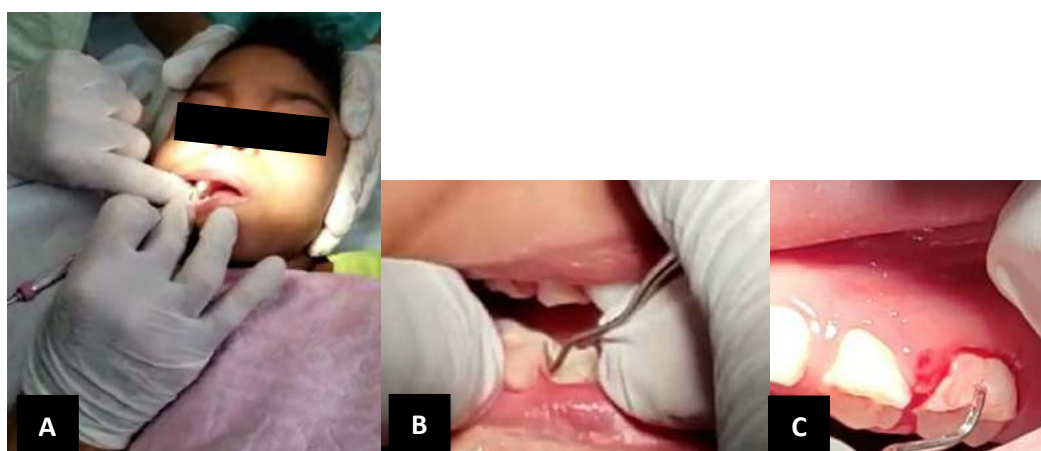


Figure 4: Dental care for a microcephaly patient. Beginning of dental calculus removal in deciduous molars (A-B). After the procedure (C)

Religious and spiritual beliefs regulate individuals' emotions and can influence the acceptance of personal or loved ones' medical diagnosis and treatment (ABUCHAIM, 2018). In order to contribute to the mental health of parents with microcephaly children, the EA Nursing team promoted a virtual meeting to dialogue with mothers about spirituality and religiosity in their daily routine. There was also the participation of the EA Dentistry team. During reports of these solidary university education with greater

patients' mothers, feelings such as faith, hope and love were perceived. This interdisciplinary link allowed students the opportunity of an ethical and understanding of the social dynamics in which they are inserted. In addition, the Nursing team contributed significantly to the development of cards and videos on preventive care against COVID-19 and the importance of vaccination (Figure 5 A-B), interacting with the students and the local community.



Figure 5: Videos about preventive care against COVID-19. Informational cards regarding vaccination importance

As for the relationship between teaching, research and extracurricular activities, the academic team showed that the EA deeply contributed to the improvement of their education. The integration of theory and practice enabled them to become professionals with social responsibility in the future. As for scientific knowledge, the EA consisted of weekly pedagogical meetings for six months by Google Meet to present seminars, discuss materials and information to be used in cards and videos. Also, there were discussions of what was prepared for the weekly exhibition, and

experience reports about interaction with families (Figure 6). Scientific productions resulted in publishing of a paper and 2 abstracts in national events. One of these was awarded second place in an experience report academic fórum. As for the research, a project was started on the facial and dental characteristics of children with microcephaly. For the interdisciplinary relation with the nursing area, an important contribution of this practice can be observed in the public health services, resulting in enhanced pedagogical processes.



Figure 6: Seminar presentation present by a student for the academic community of UES

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IV. CONCLUSION

After the social isolation period resulted from the COVID-19 pandemic, the return to clinical practices with the aim to offer oral health for children with microcephaly were enabled, without failing to provide online monitoring of preventive hygiene oral care. Parents' disbelief about the inclusion of their children in dental care must be overcome by adopting preventive and interceptive procedures within public health services for individuals with microcephaly, in order to define a realistic prognosis and develop a health care protocol. The benefits arising from lessons, research and extracurricular activities provided a permanent link between the academic community and society.

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"They are Not 'Hidden', 'Unseen' or 'Hard-To-Reach'": Reflections on Recruiting Women who Have Sex with Women to Tailored Public Health Research in Tanzania

Switbert R. Kamazima, Jackline V. Mbishi, Happiness P. Saronga & Saidah S. Bakar

ABSTRACT

Available literature suggests that women who have sex with women (WSW) are 'hidden', 'unseen', or 'hard-to-reach' because they operate underground, their size is unknown, and their daily lived experiences remain a mystery to outsiders including (public) health researchers. As a result, WSW are claimed unwilling to participate in medical/clinical or public health research. In this paper, we present our experience recruiting WSW to tailored public health research in Tanzania: the planned and unique nationwide integrated socio-behavioral and biological surveillance survey. We conducted a cross-sectional descriptive and retrospective qualitative formative study in Dar-es-Salaam Region, Tanzania, between January and February, 2021. Study population included community leaders; and WSW aged 18 years and above, who had lived in Dar-es-Salaam for six (6) months or more; had engaged in same-sex sex in the past year or were in same-sex relationship(s); had knowledge of WSW's lived experiences, and willing to participate in the study. Findings indicate that WSW in the study area are not 'hidden', 'unseen' or 'hard-to-reach' as researchers and other professionals claim.

Keywords: women's sexuality; 'hidden', 'unseen', or 'hard-to-reach' population; women who have sex with women; public health research; qualitative field research; Tanzania.

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“They are Not ‘Hidden’, ‘Unseen’ or ‘Hard-To-Reach’”: Reflections on Recruiting Women who Have Sex with Women to Tailored Public Health Research in Tanzania

Switbert R. Kamazima^α, Jackline V. Mbishi^σ, Happiness P. Saronga^ρ & Saidah S. Bakar^ω

ABSTRACT

Available literature suggests that women who have sex with women (WSW) are ‘hidden’, ‘unseen’, or ‘hard-to-reach’ because they operate underground, their size is unknown, and their daily lived-experiences remain a mystery to outsiders including (public) health researchers. As a result, WSW are claimed unwilling to participate in medical/clinical or public health research. In this paper, we present our experience recruiting WSW to tailored public health research in Tanzania: the planned and unique nationwide integrated socio-behavioral and biological surveillance survey. We conducted a cross-sectional descriptive and retrospective qualitative formative study in Dar-es-Salaam Region, Tanzania, between January and February, 2021. Study population included community leaders; and WSW aged 18 years and above, who had lived in Dar-es-Salaam for six (6) months or more; had engaged in same-sex sex in the past year or were in same-sex relationship(s); had knowledge of WSW’s lived experiences, and willing to participate in the study. Findings indicate that WSW in the study area are not ‘hidden’, ‘unseen’ or ‘hard-to-reach’ as researchers and other professionals claim. Similarly, we demonstrate that WSW’s recruitment to participate in (public health) research is not as tedious and tricky as previously presented. We validate that WSW exist in Tanzania and are willing to participate and support research with direct relevance to their livelihoods and their community’s wellbeing. They require WSW-friendly and sensitive approaches, as well as working with or through their trusted individuals, institutions, or organizations.

Keywords: women’s sexuality; ‘hidden’, ‘unseen’, or ‘hard-to-reach’ population; women who have sex with women; public health research; qualitative field research; Tanzania.

Author α ρ: Behavioral Sciences Department, School of Public Health and Social Sciences, Muhimbili University of Health and Allied Sciences, P.O. Box 65001, Dar-es-salaam, Tanzania.

σ: Epidemiology and Biostatistics Department, School of Public Health and Social Sciences, Muhimbili University of Health and Allied Sciences, P.O. Box 65001, Dar-es-salaam, Tanzania.

ω: Community Health Department, School of Public Health and Social Sciences, Muhimbili University of Health and Allied Sciences, P.O. Box 65001, Dar-es-salaam, Tanzania.

I. INTRODUCTION

A review of available literature indicates that women who have sex with women (WSW) are a minority group; constituting a small proportion of a larger group of lesbians, gay, bisexual, transgender and intersex (LGBTI) that are wildly overestimated [1]. This perspective, suggests WSW are another ‘hidden’, ‘unseen’ or ‘hard-to-reach’ group like injecting drug users (IDUs), men who have sex with men (MSM), female sex workers (FSWs), and human and organ traffickers (TIP/O) whose actual sizes are unknown and tricky to estimate. In addition, these groups are known engaging in subversive behaviors and practices that lead to stigma, rejection, all forms of violence, discrimination, and criminalization from governments, societies, and communities around them [2,3]. In turn, this situation limits their willingness to participate in studies targeting them or the general population; thus, posing a

significant obstacle to recruitment [4-6] and underrepresentation in (health) research [7].

However, some researchers who have successfully conducted studies among minority groups observe that failure to recruit participants from diverse pools reflects not making enough effort to be inclusive. In other words, it justifies that participating in medical/clinical or public health research in the developed countries (the U. S. A. and Europe) is a matter of “who is invited to participate rather than who is willing to” [4], leading to underrepresentation of minority groups in medical/clinical trials and public health interventions [8]. In this paper, we present our experiences recruiting WSW to participate in the first and unique study (the formative qualitative, Phase I) among WSW in Tanzania’s largest and commercial city, Dar-es-Salaam. The main goal of this study was to generate data to inform the planned nationwide integrated biological and socio-behavioral survey (Phase II). We present and argue that recruitment of WSW for this study was not as difficult as we, the researchers, and colleagues at the Muhimbili University of Health and Allied Sciences (MUHAS) anticipated. We further point out some factors that could have contributed to this experience/scenario.

II. STUDY AREA, METHODS AND PARTICIPANTS

We conducted a cross-sectional and retrospective qualitative formative study with WSW in three study districts of Dar-es-Salaam region: Temeke, Kinondoni, and Ilala. We purposely chose Dar-es-Salaam City because it is Tanzania’s largest and commercial city, harboring persons from different backgrounds and engaging in varied health behaviors and practices. Study population included community leaders; and WSW aged 18 years and above, who had lived in Dar-es-Salaam for six (6) months or more; had engaged in same-sex sex in the past year or were in same-sex relationship(s); had knowledge of WSW’s lived experiences, and willing to participate in the study.

The MUHAS Institutional Review Board (IRB) reviewed the study protocol and granted ethical

clearance. The Dar-es-Salaam Regional Administrative Secretary (RAS), the Ilala, Kinondoni, and Temeke District Administrative Secretaries (DAS), and the Street authorities granted permission to conduct the study in their respective areas. The process of interviewing neither had harm to nor re-traumatized the study participants. The average duration of IDIs and FGDs was one and half hours. However, as our participants had interest in this study, some IDIs and FGDs took longer time. The aim was to understand what it means to be a WSW in Tanzania to inform future female same-sex public health research targeting this group.

We selected and trained our research assistants (RAs) for three days on the study objectives and process, the vulnerability of WSW, ethical issues around this sensitive study, and proper interaction/interviewing procedures with the study participants. With permission from the participants, FGDs and IDIs were audio-recorded. In addition, the RAs took field notes and wrote full reports of observations on the same day. FGDs and IDIs were conducted in Kiswahili, a national language understood and spoken by almost everybody in the study area. We transcribed and translated data, followed by data analysis applying thematic approach where open systematic coding of data in the participants’ language and combining emerging emic concepts with preconceived theoretical constructs was used.

2.1 ‘Hidden’, ‘unseen’ and ‘hard - to - reach’ populations defined

Researchers use the term ‘hidden’ to refer to groups that have no defined boundaries, size and are tricky to sample [9]. ‘Unseen’ populations are claimed nonexistent, and if they do, are too small to impact the system in question or minority groups highly underrepresented in research [10-12]. Other researchers describe a population that is difficult to access as ‘hard-to-reach’ [13,14]. In this context, WSW may be defined ‘hidden’ because their size is unknown, their lived experiences are mysterious and work underground due to illegal status of their same-sex behaviors and practices; ‘unseen’ due to their survival tactics in communities where their overt

nature could put them at risk of greater harm (such as: discrimination, criminalization, stigma, violence, rejection and abuse); and ‘hard-to-reach’ due to the unjustified belief that WSW would be unwilling to engage (sharing their lived experiences) with outsiders, including (public health) researchers. It should be noted, however, that though the groups are outwardly distinct, the three terms are used interchangeably to denote vulnerable groups subjected to discrimination, rejection, and stigma [15] in referred contexts.

2.2 WSW as defined in this study project context

In this study project, we adopted and use an inclusive concept/term ‘women who have sex with women’ (WSW) [16] to refer to the targeted population, “Women who engage in sexual activities with other women, whether or not they identify themselves as lesbians, bisexual, pan-sexual, heterosexual, or discipline with sexual identification altogether” [17]. We preferred this definition because we focus on WSW’s behaviors and practices rather than labels attached to WSW’s behaviors and practices [18] and the public health implications of female same-sex relationships that develop from intimacy or sexual/physical attraction. As observed in previous studies, “Not all women who have sex with women are lesbians ... They might identify as straight, bi, span, queer, gay or curious ... They might be cis gendered, trans or non-binary [19,20]. Moreover, it is known that “Women who don’t identify as lesbians, bisexual, queer or even questioning often have had sexual relationships with other women” [21].

2.3 Female same-sex relationships and activities are illegal in Tanzania: the study context

The Tanzania Constitutions (Mainland and Zanzibar) do not recognize same-sex relationships. Thus, same-sex relationships/couples have no recognition on Tanzania Mainland (The Tanzania Penal Code of 1945 as revised by the *Sexual Offences Special Provisions Act, 1998*) and Zanzibar (The Zanzibar Penal Code of 1934, as amended in 2004). Same-sex behaviors and practices, therefore, are crimes punishable on conviction by life imprisonment [22,23].

Consequently, receiving death threats and persecutions is normal for LGBT individuals making it a matter of survival to keep their homosexuality hidden and rarely reported on [3,16,24]. Violence, rape, social exclusion (denial, rejection, stigma, and isolation), and discrimination characterize the daily life of individuals engaged in same-sex relationships in this country [3]. This situation has adverse effects to the WSW’s health, healthcare and livelihoods [3,24,25] triggering the horror of facing difficulties recruiting study participants from this group.

III. STUDY TEAM STRENGTHS

The investigators on this study project are PhD/Master/First degree holders and members of the School of Public Health and Social Sciences (SPHSS) in the Behavioral Sciences, Epidemiology and Biostatistics, Community Health, and Environmental and Occupational Health Departments at MUHAS, vested with various interdisciplinary research skills and methods. The team has accumulated rich experience (from 1994 to date) studying issues and groups in this country whose behaviors and practices are socially regarded taboos, illegal, crimes and generally considered ‘un-African’: FSWs (sex work), IDUs (injecting drugs), human traffickers [trafficking in persons/organs (TIP/O)], homosexuality (male same-sex sex, MSM), borders, borderlands and borderlanders (border crossing practices), and now, WSW (female same-sex sex). A challenge we have always faced is proving to the study protocol reviewing bodies on the possibility of recruiting a ‘representative sample size’ of the group in question to participate in the study. So, we wished to have this justification from our formative research that recruiting WSW for a nationwide behavioral and biological surveillance survey (among WSW) is possible in Tanzania. Experience we have gained from previous research activities, facilitated navigating and negotiating rapport among our study populations.

3.1 Pre-study consultations

Upon receipt of research clearance from MUHAS IRB, we immediately started conducting initial

formal and informal consultations with colleagues at MUHAS and beyond. The aim was to solicit perceptions they had on our study and approach. On the one hand, most of our colleagues were pessimistic that we could succeed recruiting such women in the country. One fellow staff, for example, observed, *“I don’t think there is any Tanzanian woman or girl that would identify herself a lesbian or having sex with other women ... You should know, same-sex sex is illegal in this country ... Hence, if at all female same-sex behaviors exist, they are conducted underground and unnoticed by the outsiders ... However, try your luck and best wishes”* (Colleague 1_MUHAS, 2021). Another colleague commented, *“You better go to Mara region where women marry women known as ‘Nyumba ntobu’... However, this traditional ritual is fading ... If you are lucky, you may find very few of them”* (Colleague 2_MUHAS, 2021). The other colleague observed, *“Are you among those promoting homosexuality in this country? ... How different are those women [WSW] from heterosexual [women] to call for special attention from the public?”* (Colleague 3_MUHAS, 2021).

On the other hand, colleagues who had conducted a behavioral and biological survey using respondent driven sampling (RDS) technique [26] among MSM in the country, had encouraging perspectives. One of them, for example, said, *“We have learnt from our study group [MSM] that there are many WSW in Dar-es-Salaam and other urban centers in this country ... All you need is to have a proper entry point that we shall link you to”* (Colleague 4_MUHAS, 2021). Similarly, from our search on the internet, we found contacts of LGBTs group established in 2009 with a cause for other LGBTs in Tanzania – the LGBT Voice Tanzania: location, telephone numbers, and email, that we used to seek audience with the NGO’s officials and members.

3.2 Initial contacts with the targeted population

We held initial meetings with the Executive Director of the LGBT Voice Tanzania and our contact persons known living in same-sex unions or having sex with fellow women. At these meetings, we introduced the study protocol and

the expectations we had: learning from the WSW’s live-experience and their (health) needs to generate information needed for Phase II of the study project (the nationwide integrated biological and socio-behavioral survey). In turn, information generated from the survey would facilitate improving healthcare professionals’ ability to diagnose, treat, control, and prevent illnesses among WSW and the general public in Tanzania.

During the same meetings, the LGBT Voice Tanzania Director and the self-identifying WSW highlighted the ‘dos’ and ‘don’ts’ we must observe when interacting with members of this group. They assured us (the research team) that there were so many WSW of different backgrounds that we should be prepared to encounter and support throughout our study project (the two phases). In addition, they provided us with recommendations and solutions to potential challenges we were likely to face in this course. Our initial WSW contact person, for example, stressed, *“For you to succeed, you should work through and with us to reach these women ... Failure of which, you will end up in frustration”* (Informal interviews–MUHAS, 2021) 1).

3.3 Selection, characteristics and training of research assistants

Based on recommendations from our initial contacts with members of the targeted population, we selected nine (9) female research assistants (RAs) with a minimum education of a first degree in social sciences, good experience in conducting field research, and with good interviewing and probing skills. The RAs were among those we had worked with before on other studies [the baseline human trafficking study (in 2009-2010), the HIV behavioral and biological surveillance survey among FSWs in Dar-es-Salaam, 2010, and cross-border cooperation along the Tanzania-Uganda border (2002 and 2017/2018)]; have good experience managing field research logistical issues; and capable of working under minimum supervision. The RAs underwent a three days’ training on the objectives and procedures for this study. In addition, the RAs were sensitized on the vulnerability of WSW, exposed to the proper

“They are not ‘hidden’, ‘unseen’ or ‘hard-To-Reach’”: Reflections on Recruiting women who Have Sex With Women to Tailored Public Health Research in Tanzania

interaction and interviewing procedures/ethics with the study participants, and alerted on potential challenges they were likely to face in the field and possible mitigation and solutions.

3.4 (Always) expect the unexpected

With all the study logistics under control, we planned to start field work during the last week of March, 2020. However, by mid-March, Coronavirus 2019 disease (COVID-19) had been identified in the country on March 16. The government hastily closed learning institutions, banned activities and events that could result in crowding (sports, rallies, and cultural events like weddings) for the public health good [27-30]. Following the government order, the National Health Research Ethics Committee (TANHER Committee), the national overseer of health research activities in the country issued a directive banning all research activities (except lab-based and clinical trials) till further notice! Our team had to abide by this ban. However, the researchers remained digitally in touch with initial contacts. Most important, perhaps, is that the study funding tenure was one year, that is, September 2019 to August 2020! Anxiety of not meeting the study deadline grew among the research team members.

On June 16, 2020, the government announced recommencing of all activities that were suspended as part of the response to COVID-19 from Monday, June 29, 2020. Following the government's decision, the TANHER Committee called for the resumption of research activities in the country. Nevertheless, fear of being infected or spreading the coronavirus remained high among the researchers and members of the community. Our team, however, resumed activities in December. The research clearance application process at the Dar-es-Salaam regional level was completed in early January 2021, allowing the team to proceed to the district level. We started interacting with our study participants during the second week of January 2021.

Through our contact persons, recruitment of eligible study participants became somehow easier than the research team expected. By the

end of February, 2021, we had conducted six (two in each district) focus group discussions (FGDs) with WSW; 24 in-depth interviews (IDIs) with eight (8) key informants (KIs) (where each KI was interviewed at least three times). In addition, we conducted seven (7) IDIs with members of the community. As indicated earlier, all FGDs and IDIs were audio-recorded and the average time for each IDI and FGD was one and half hours. However, as our participants had interest in this study, some IDIs and FGDs took longer time. Detailed information on the WSW's existence and willingness to participate in future (public) health research is provided by Kamazima, *et al.*, [16] and Kamazima, *et al.*, [30] respectively. It suffices to note here that recognizing, respecting and considering WSW's life-norms and conditions in study researches' protocols, opens opportunities to maximize WSW's visibility by targeting recruiting approaches capturing women with differentiated characteristics and backgrounds to increase their willingness to participate in (public) health research.

3.5 What made participants' recruitment quick and successful?

We are contented, we were able to recruit, interact with adequate number of WSW, and generate information needed for this formative study on time due to a number of factors including: 1) the rich research knowledge, skills, and experience the investigators and RAs have facilitated building trust and acceptance among the initial contacts and the study participants at large. Being a MUHAS-based study (a trusted research institution and known conducting studies with vulnerable populations), was an added advantage to this study; and 2) working through and with persons and organizations trusted by the WSW community granted a wide range of opportunities to the research team's access to the study population and the women's willingness to participate in the study.

In addition, as the WSW we studied reported [30], the WSW were willing to participate and share their lived-experiences with the research team; recruit fellow WSW to the FGDs, and ready to participant in Phase II of the study due, but not

limited, to: 1) Phase I researchers approached the WSW with sensitivity, comprehensively explained the importance of participating in both phases of the study project, and clarified what participation in the study project entails; 2) the research team demonstrated love, trust, respect, and sympathy to the group irrespective of their behaviors and practices; 3) interactions with the research team enlightened the WSW on some female same-sex issues (female same-sex sex protective devices, female same-sex law-related matters, and female same-sex sex health-related problems) that they were unaware of [31]; 4) the study, both Phase I and II, promise relevant to the WSW's community around the country; 5) WSW recognized the study could uncover and facilitate addressing female same-sex health, reproductive health needs, and establish the group's size; and, 6) WSW believe having opportunity to access quality and equitable health and healthcare services they utterly need, could open more opportunities in that direction.

IV. CONCLUSION

Our study demonstrated that WSW are not a 'hidden', 'unseen' or 'hard-to-reach' group as many of the (public) health researchers and health professionals (may) think. WSW exist in this country and are willing to participate in research with direct relevance to their livelihoods and their community's wellbeing. They require WSW-friendly and sensitive approaches; working through and with their trusted individuals, groups, institutions, and organizations; and clear elucidations of what participation in a (public health) study or research entails. Researchers, therefore, ought to develop WSW-tailored research protocols' content, communication messages, and recruitment tactics to recognize, appreciate, and embrace the specific characteristics, backgrounds, and concerns of WSW in Tanzania.

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Toxicological Studies of Picralima Nitida Aqueous and Ethanolic Extracts in Animal Model

Oluwagbemiga O. Aina, Onyinye C. Okoyenta, Kafilat O. Kareem, Damilare J. Bamgbose, Olusola Ajibaye & Bamgboye M. Afolabi

ABSTRACT

Introduction: Picralima nitida is a seed bearing tree whose dried seeds are used in traditional medicine throughout West Africa, particularly in Ghana, Ivory Coast and Nigeria. The seeds are crushed and taken orally for the treatment of malaria, diarrhoea, pain, hypertension, jaundice, dysmenorrhoea, and gastrointestinal disorders. There is paucity of data on the toxicity and safety profile of Picralima nitida, thus the need for this study. Acute toxicity study was carried out on Picralima nitida aqueous and 80% ethanolic extract in order to determine its acute toxicity and LD₅₀.

Keywords: picralima nitida, phytochemical constituents, acute oral toxicity, histopathology, medicinal plants.

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Toxicological Studies of *Picralima Nitida* Aqueous and Ethanolic Extracts in Animal Model

Oluwagbemiga O. Aina^α, Onyinye C. Okoyenta^σ, Kafilat O. Kareem^ρ, Damilare J. Bamgbose^ω,
Olusola Ajibaye[¥] & Bamgboye M. Afolabi[§]

ABSTRACT

Introduction: *Picralima nitida* is a seed bearing tree whose dried seeds are used in traditional medicine throughout West Africa, particularly in Ghana, Ivory Coast and Nigeria. The seeds are crushed and taken orally for the treatment of malaria, diarrhoea, pain, hypertension, jaundice, dysmenorrhoea, and gastrointestinal disorders. There is paucity of data on the toxicity and safety profile of *Picralima nitida*, thus the need for this study. Acute toxicity study was carried out on *Picralima nitida* aqueous and 80% ethanolic extract in order to determine its acute toxicity and LD₅₀.

Methods: Phytochemical analysis was carried out on *Picralima nitida* to identify its active phytochemical constituents both qualitatively and quantitatively. Acute oral toxicity tests were done using female Swiss albino mice that weighed 20-23g following the OECD methods. Fixed doses of 300mg/kg, 2000mg/kg and 5000 mg/kg of *Picralima nitida* aqueous and 80% ethanolic extracts were administered to the animals once and then observed for 14days. The control group received distilled water only ad libitum. At the end of the 14days, the animals were sacrificed and analyzed for histopathological changes.

Results: The LD₅₀ of the aqueous and ethanolic extract of *Picralima nitida* were found to be ≥ 2000 mg/kg. The heart of the animals that received only distilled water, and those that received 300mg/kg, 2000mg/kg, and 5000mg/kg of *Picralima nitida* aqueous extract had no histopathological damages. The photomicrograph of the liver, kidneys, lungs of the untreated and treated groups of aqueous extract show some histopathological alterations. While, the

histologic sections of the heart, liver, kidney, and lungs of the animals that received 2000mg/kg and 5000mg/kg of ethanolic extract of *Picralima nitida* had some histopathological changes. Similar injuries were also seen in the untreated group.

Conclusion: The phytochemical screening revealed that *Picralima nitida* contains important antioxidants and other phytochemicals with various health benefits. While the acute toxicity assessment of the aqueous and 80% ethanolic extracts of *Picralima nitida* indicate that *Picralima nitida* is safe.

Keywords: *picralima nitida*, phytochemical constituents, acute oral toxicity, histopathology, medicinal plants.

Author α σ ρ ω ¥ : Centre for Research in Traditional, Complementary and Alternative Medicine, Biochemistry and Nutrition Department, Nigerian Institute of Medical Research (NIMR), 6, Edmund Crescent, P.M.B 2013, Yaba, Lagos, Nigeria.

§: Health, Environment and Development Foundation (HEENDEF), 18 Ogunfunmi Street, Surulere, Lagos, Nigeria. African, Pan-African Health and Collaborative, Salisbury, North Carolina, USA.

I. INTRODUCTION

Medicinal plants are often used in the treatment of various ailments in traditional and complementary medicine in many parts of the world [1, 2]. *Picralima nitida* (Eso Aberé) is a plant genus in the family Apocynaceae, first described as a genus in 1896. It contains only one known species, *Picralima nitida*, native to tropical Africa (Benin, Ghana, Ivory Coast, Nigeria, Gabon, Cameroon, Cabinda, Central African Republic, Republic of Congo, Zaire, Uganda) [3, 4]. *Picralima nitida*, the akuamma, is a tree. The

dried seeds from this plant are used in traditional medicine throughout West Africa, particularly in Ghana as well as in the Ivory Coast and Nigeria. The seeds are crushed or powdered and taken orally, and are mainly used for the treatment of malaria, diarrhoea, pain, hypertension, jaundice, dysmenorrhoea, gastrointestinal disorders [5, 6]. *Picralima nitida* seeds contain a mixture of alkaloids producing antipyretic and anti-inflammatory effects along with analgesia in animal studies [7].

Most synthetic anticancer drugs that are currently available are highly expensive, elicit serious and toxic side-effects and mostly affordable by the rich and elite. Also, they are not readily available to the majority of the African population. Hence, researchers are currently working assiduously on herbal preparations and crude extracts from medicinal plants to be used wholly or as an alternative complementary therapy. This study aims to investigate the safety of *Picralima nitida* aqueous and 80% ethanolic extract in animal model through acute oral toxicity study in order to determine its toxicity characteristics and LD₅₀ for subsequent *in vivo* therapeutic efficacy evaluation of *Picralima nitida* for anti-diabetics and anti-cancer activities in order to help validate drug candidates for clinical evaluation.

II. METHODS

2.1 Preparation of herbal extracts

Aqueous and ethanolic extracts of *Picralima nitida* plant were prepared according to standard methods by using distilled water and 80% ethanol respectively and the extracts obtained were then evaporated to dryness in electric oven at 40°C.

2.2 Phytochemical Analysis

Phytochemical analysis was carried out on *Picralima nitida* to identify the active phytochemical constituents both qualitatively and quantitatively. The phytochemical screenings were performed on the medicinal plants using standard procedures described elsewhere [8, 9].

2.3 Acute Oral Toxicity screening

Acute oral toxicity tests were done using female Swiss albino mice with an average weight of

20-23g. The toxicity study was done according to the OECD methods for acute oral toxicity [10]. They were obtained from the institutional animal house, Department of Biochemistry and Nutrition, Nigerian Institute of Medical Research (NIMR). The female mice used were nulliparous and non-pregnant. The animals were given standard animal pellets (Ladokun Feeds, Ibadan) and tap water *ad libitum*. The mice were maintained at a room temperature of 25 ± 3°C and a 12 h light/dark cycle. The experimental Protocol was approved by the Institutional Animal Care and Use Committee (IACUC), Department of Biochemistry and Nutrition, Nigerian Institute of Medical Research (Ethics No. IRB/17/043).

The body weight of animals was recorded individually for calculating proper treatment dosage before the test. The doses used were 300mg/kg, 2000mg/kg and 5000mg/kg body weights (b.w) of *Picralima nitida* aqueous and 80% ethanolic extracts. Animals were administered once with the different doses of the extracts. The control group received distilled water only *ad libitum*. Animals were observed for signs of toxicity in the first four hours, then at thirty minutes intervals for the next twenty-four hours. Subsequently, they were observed daily for fourteen days for any delayed toxicity. Mortality, food consumption and water intake, as well as observation for general toxicity signs were monitored and recorded daily throughout the study.

2.4 Histopathological Analysis

The animals were sacrificed by cervical dislocation after blood collection. Vital organs (liver, kidneys, lungs, and heart) were removed through a midline incision in the mice's abdomen. The organs were subjected to histopathological examination. They were fixed in 10% buffered formalin, routinely processed and embedded in paraffin wax. Paraffin sections (5 µm) was cut on glass slides and stained with hematoxylin and eosin. An experienced pathologist conducted the analysis. The slides were examined under a light microscope under ×100 magnification.

III. RESULTS

3.1 Phytochemical constituents

The results of the phytochemical evaluations of *Picralima nitida* are presented in tables 1 and 2.

Table 1: Qualitative analysis report for *Picralima nitida* phytochemical constituents

Active Phytochemicals	Inference
Flavonoids	+
Terpernoids	+
Cardiac glycosides	-
Tannins	+
Phlobatanin	-
Steroid	-
Saponin	+
Alkaloids	+
Phenol	+
Anthraquinone	-

+ means positive – means negative

Table 2: Quantitative analysis report for *Picralima nitida* phytochemical constituents

Active Phytochemicals	Quantity
Flavonoids	36%
Tannins	15.08%
Saponin	51.5%
Alkaloids	20%
Phenol	45.78mg GAE/100g

+ means positive – means negative

3.2 Acute Systemic Toxic effects

In this study, the LD₅₀ of the aqueous and 80% ethanolic extract of *Picralima nitida* were found to be ≤ 2000mg/kg body weight.

histopathological alterations. The cause of the adverse effects is unknown.

3.3 Histopathological Effect of *Picralima nitida* Aqueous extracts

The histologic structures of the vital organs (kidney, liver, lungs and heart) of the control group (untreated animals) and treated groups are shown in figures 1 to 4.

The heart of the animals that received only distilled water, and those that received low dose (300mg/kg), mid dose (2000mg/kg), and high dose (5000mg/kg) of *Picralima nitida* aqueous extract had no histopathological damages (figure 1). These indicate that the aqueous extract of *Picralima nitida* is safe on the heart even at the highest dose. However, the photomicrograph of the liver, kidneys, lungs of the untreated (control) and treated groups of aqueous extract show some

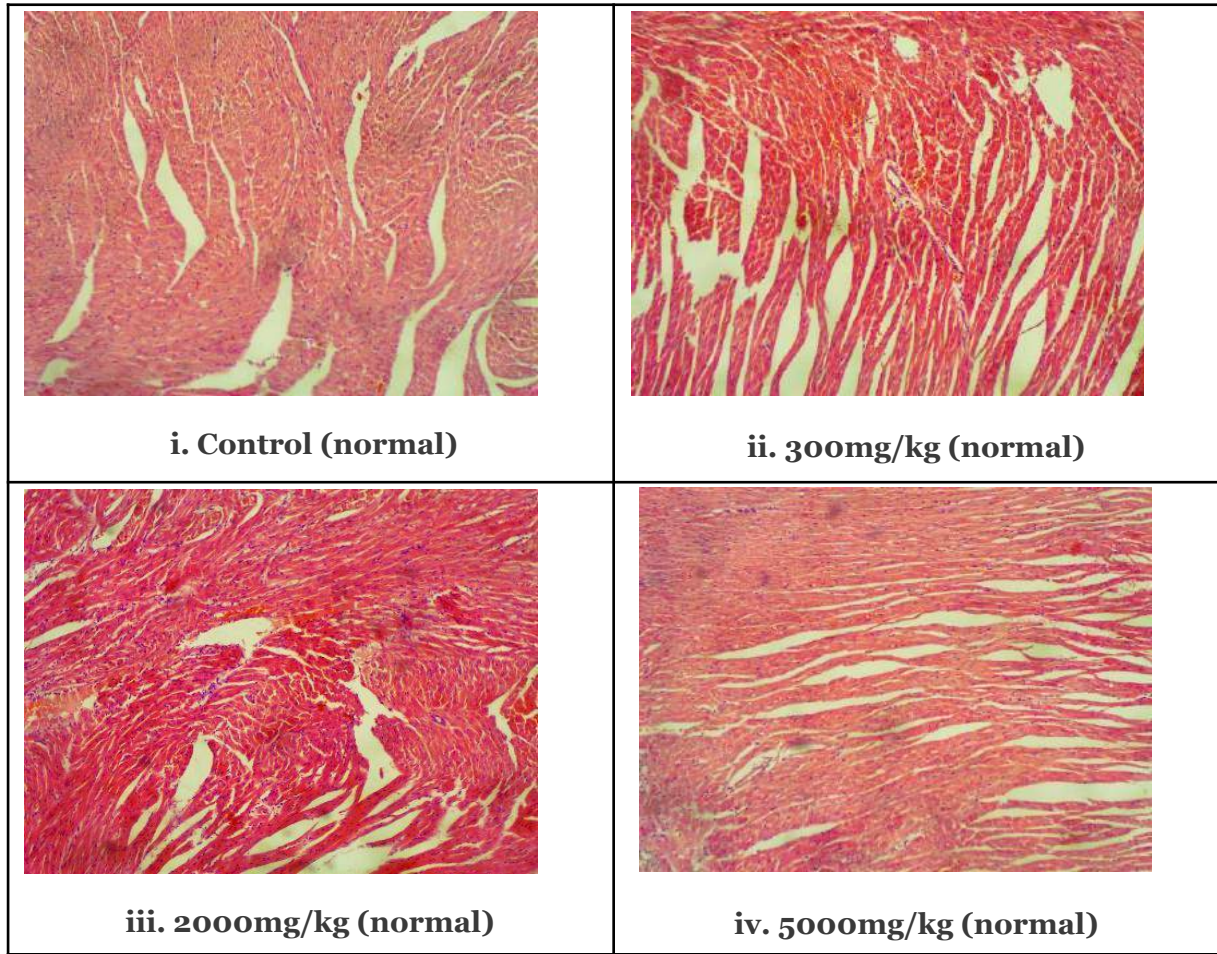
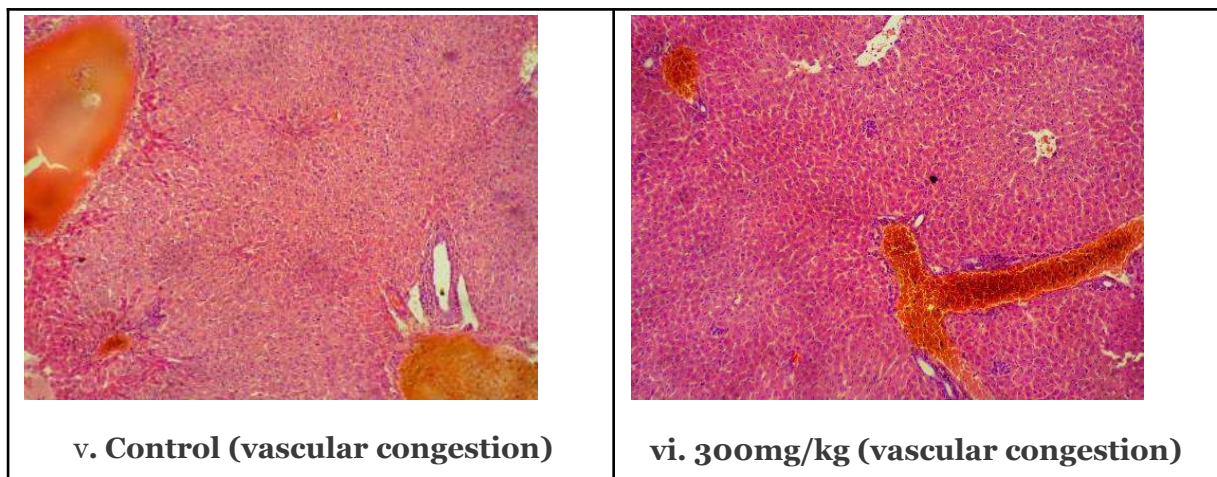


Figure 1: Histologic sections of heart excised from animals that received only distilled water (control) and those that received 300-5000mg/kg of aqueous extract of *Picralima nitida* show normal interlacing fascicles of cardiac myocytes/ myocardial cells



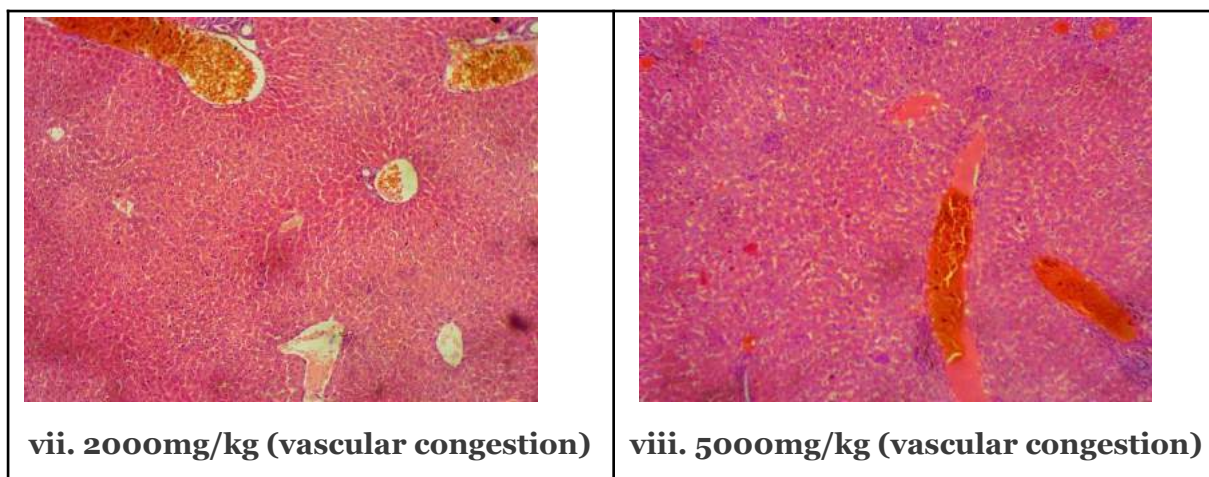


Figure 2: Histologic sections of liver excised from animals that received only distilled water (control) and those that received 300-5000mg/kg of aqueous extract of *Picralima nitida*. The liver tissue show parallel radially arranged plates of hepatocytes with the portal space and periportal zone filled with a smooth to slightly floccular pink fluid material common with edema and congested aggregates of red blood cells were also seen in both control and treated group

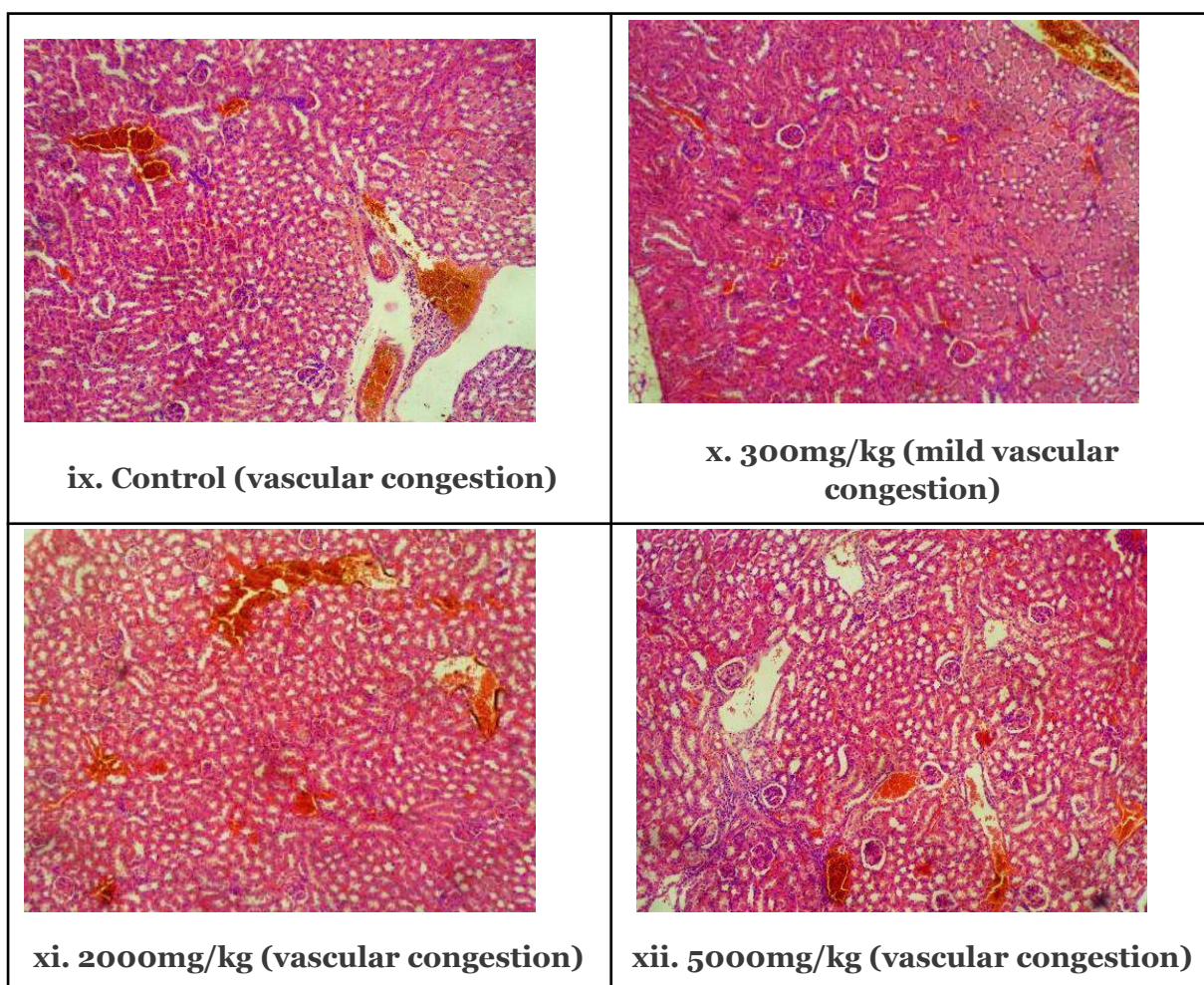


Figure 3: Histologic sections of kidney excised from animals that received only distilled water (control) and those that received 300-5000mg/kg of aqueous extract of *Picralima nitida*. Kidney tissue show normocellular glomerular tufts disposed on a background containing viable tubules. Congested blood vessels were seen

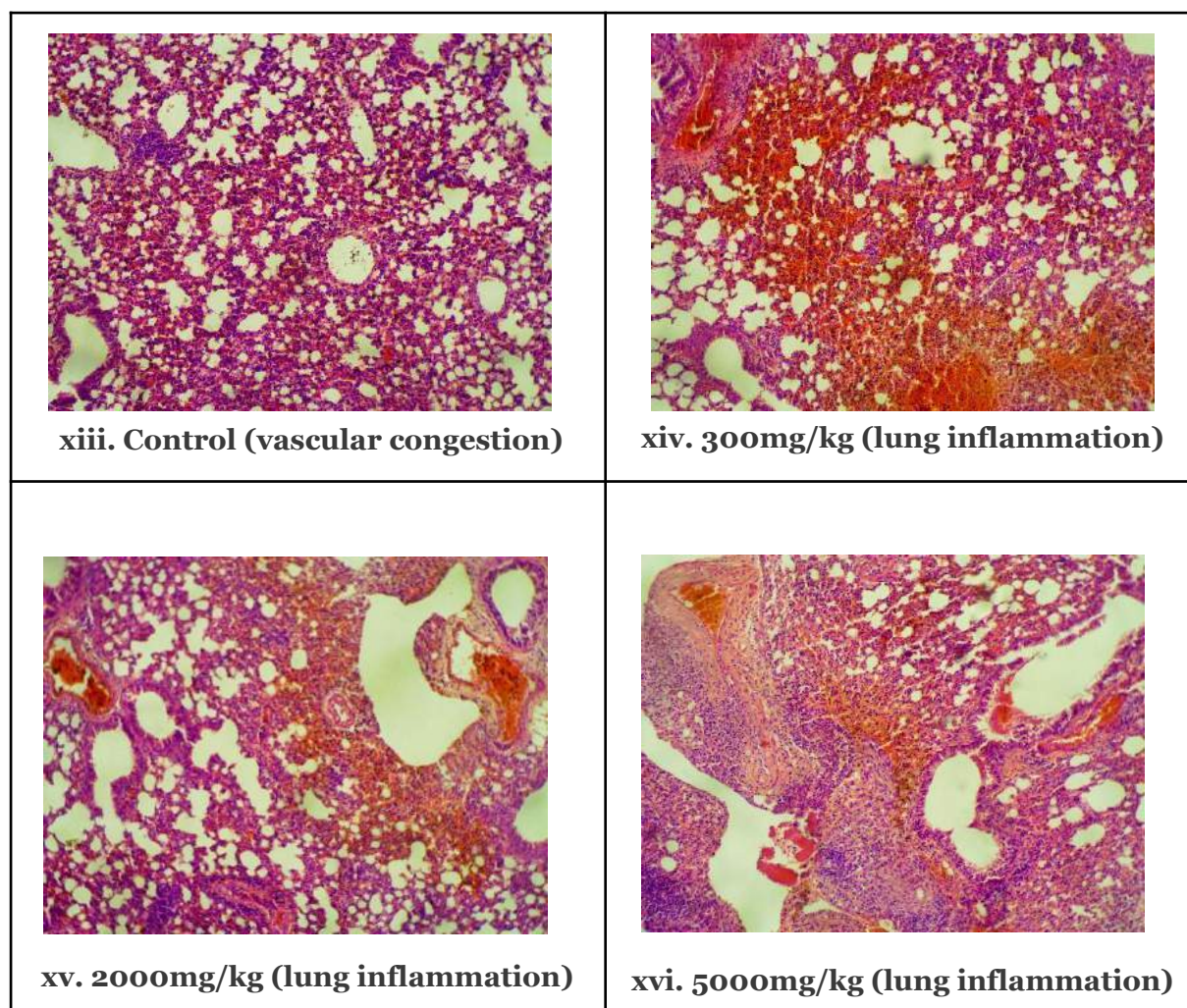


Figure 4: Histologic sections of lungs excised from animals that received only distilled water (control) and those that received 300-5000mg/kg of aqueous extract of *Picralima nitida*. Lung tissue show some alveolar filled air spaces, the vessels in the walls of the alveoli are distended and the capillaries are congested with aggregates of many red blood cells

3.4 Histopathological Effect of *Picralima nitida* 80% Ethanolic extracts

The histologic structures of the vital organs (kidney, liver, lungs and heart) of the control group (untreated animals) and treated groups are shown in figures 5 to 8.

The histologic sections of the heart, liver, kidney, and lungs of the animals that received mid dose (2000mg/kg), and high dose (5000mg/kg) of 80% ethanolic extract of *Picralima nitida* had some histopathological changes. Similar injuries were also seen in the untreated group (control) thus the cause of the histopathology is unclear.

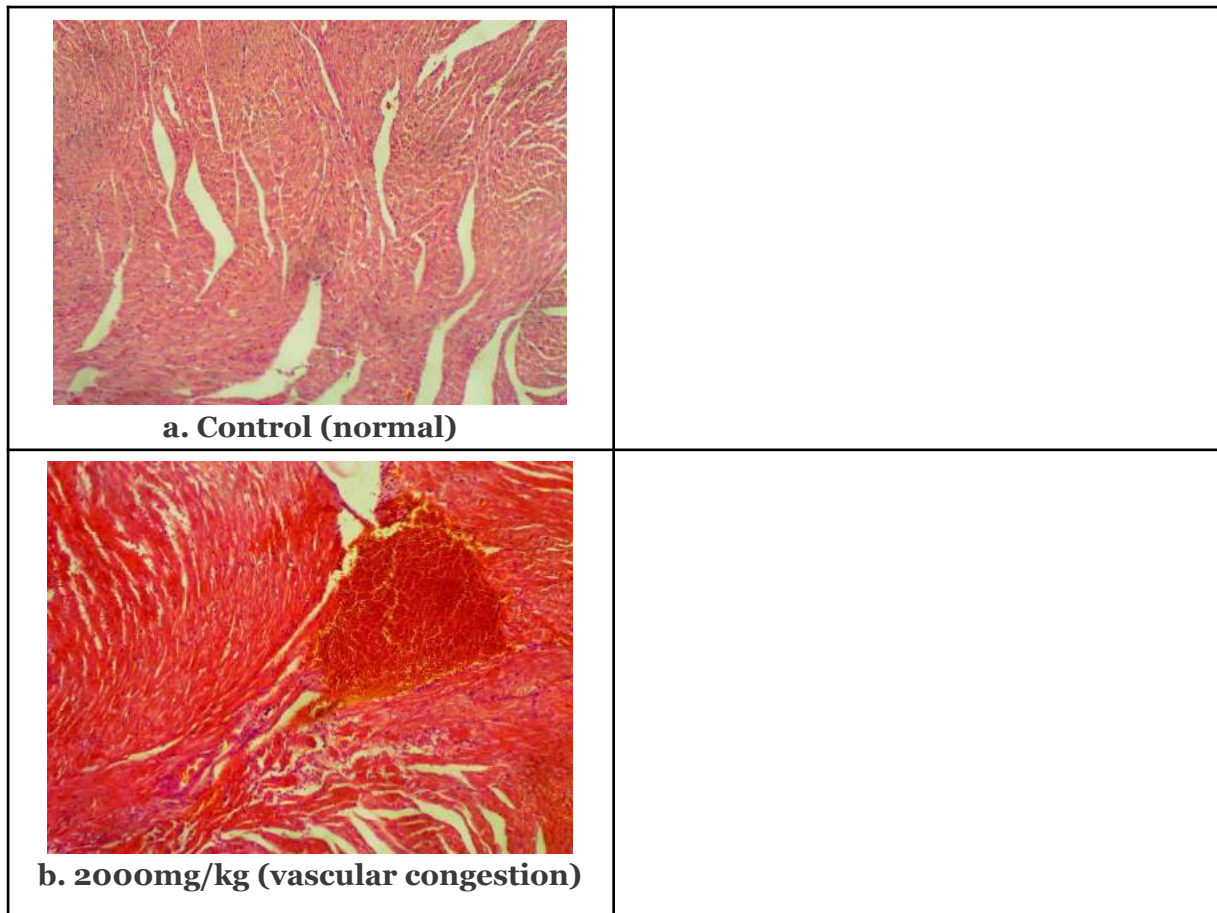
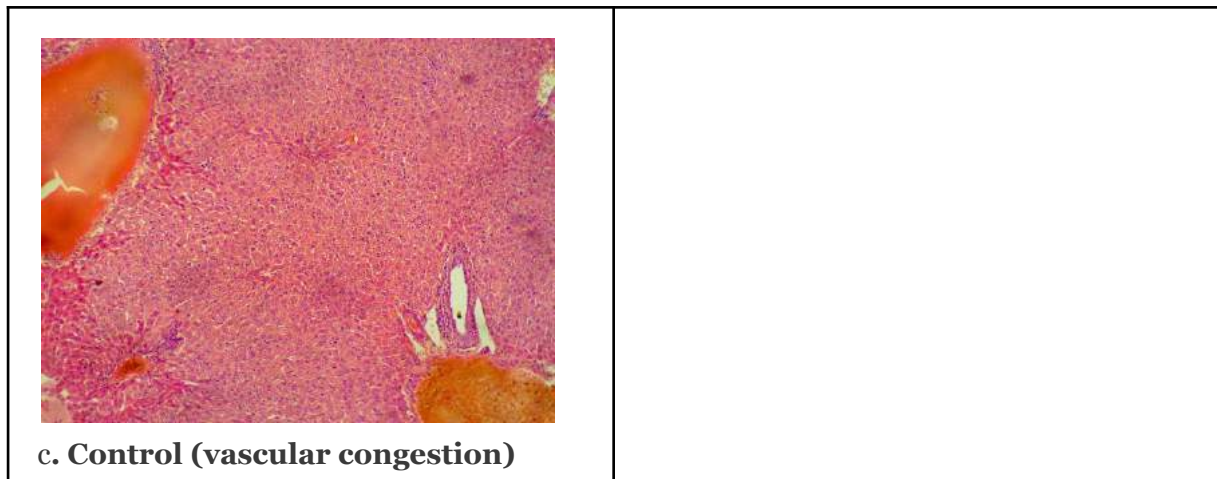


Figure 5: Histologic sections of heart show interlacing fascicles of cardiac myocytes/ myocardial cells. The heart sections for untreated and those treated with 300-5000mg/kg of ethanolic extract of *Picralima nitida* were normal



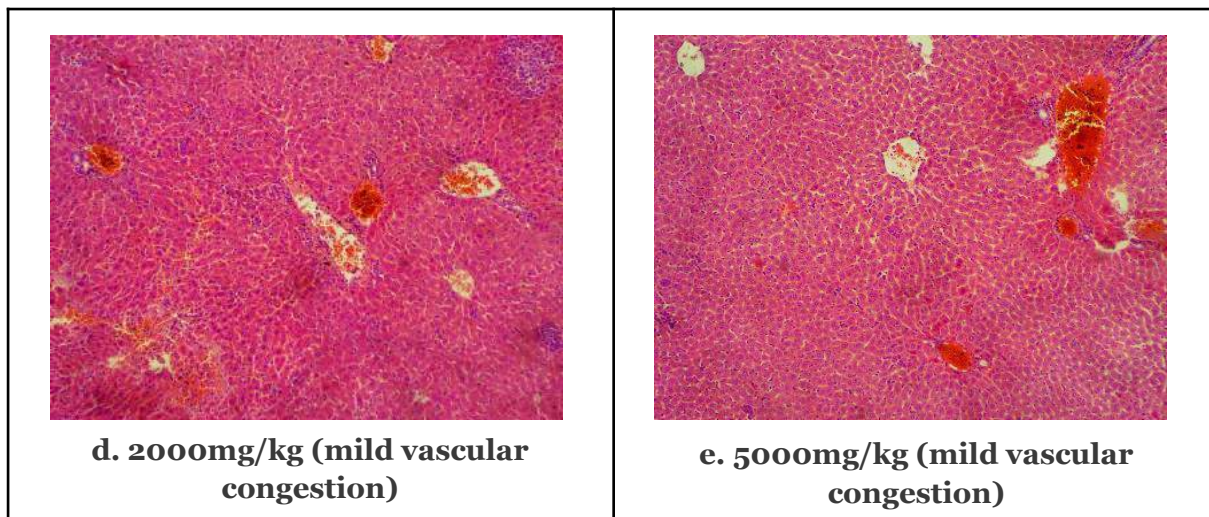


Figure 6: Histologic sections of liver tissue show parallel radially arranged plates of hepatocytes with the portal space and periportal zone filled with a smooth to slightly floccular pink fluid material common with edema and congested aggregates of red blood cells also seen. The liver sections for untreated and those treated with 300-5000mg/kg of ethanolic extract of *Picralima nitida* had mild to severe vascular congestion

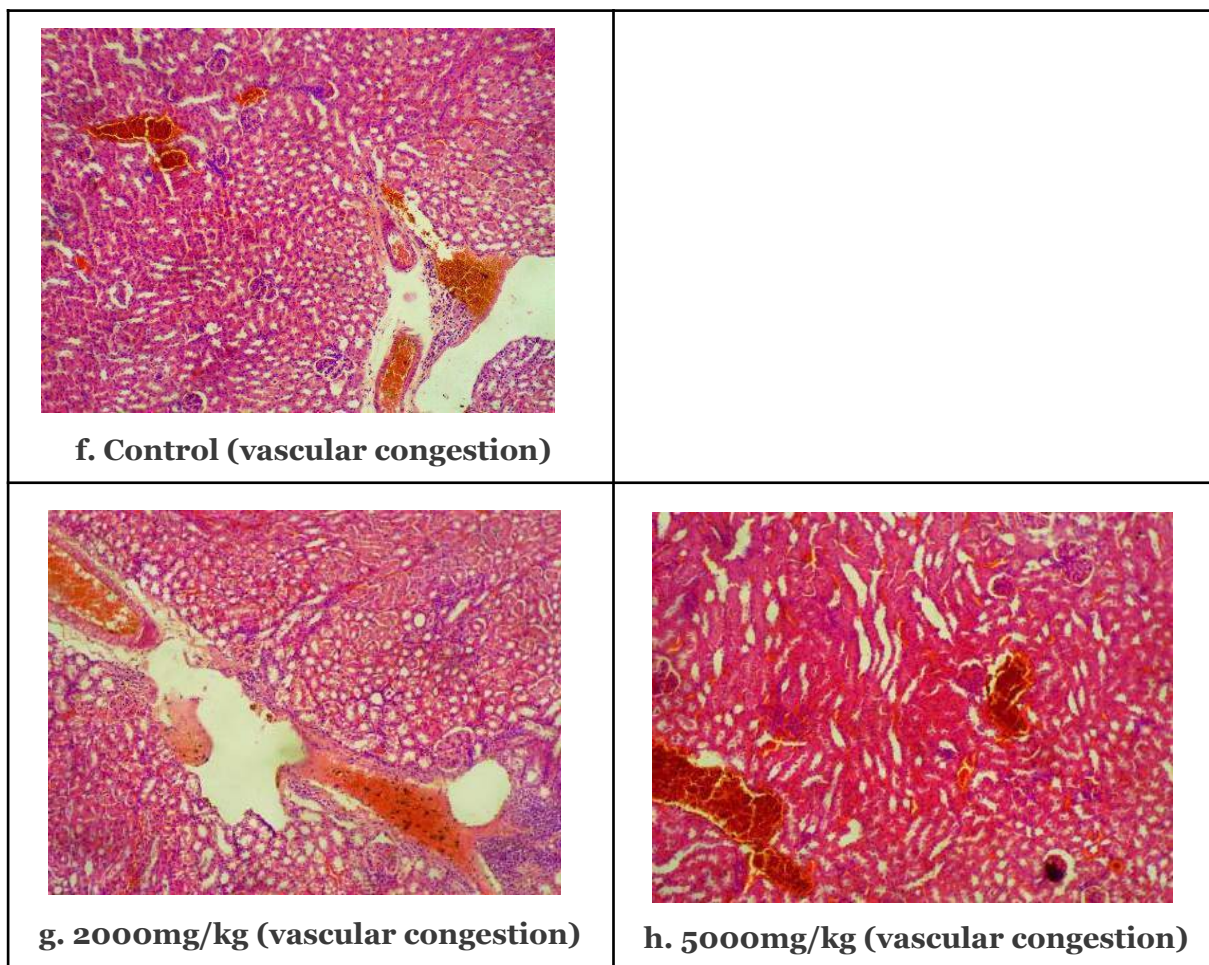


Figure 7: Histologic sections of kidney tissue show normocellular glomerular tufts disposed on a background containing viable tubules. Congested blood vessels are seen. The kidney sections for untreated and those treated with 300-5000mg/kg of ethanolic extract of *Picralima nitida* had mild to severe vascular congestion

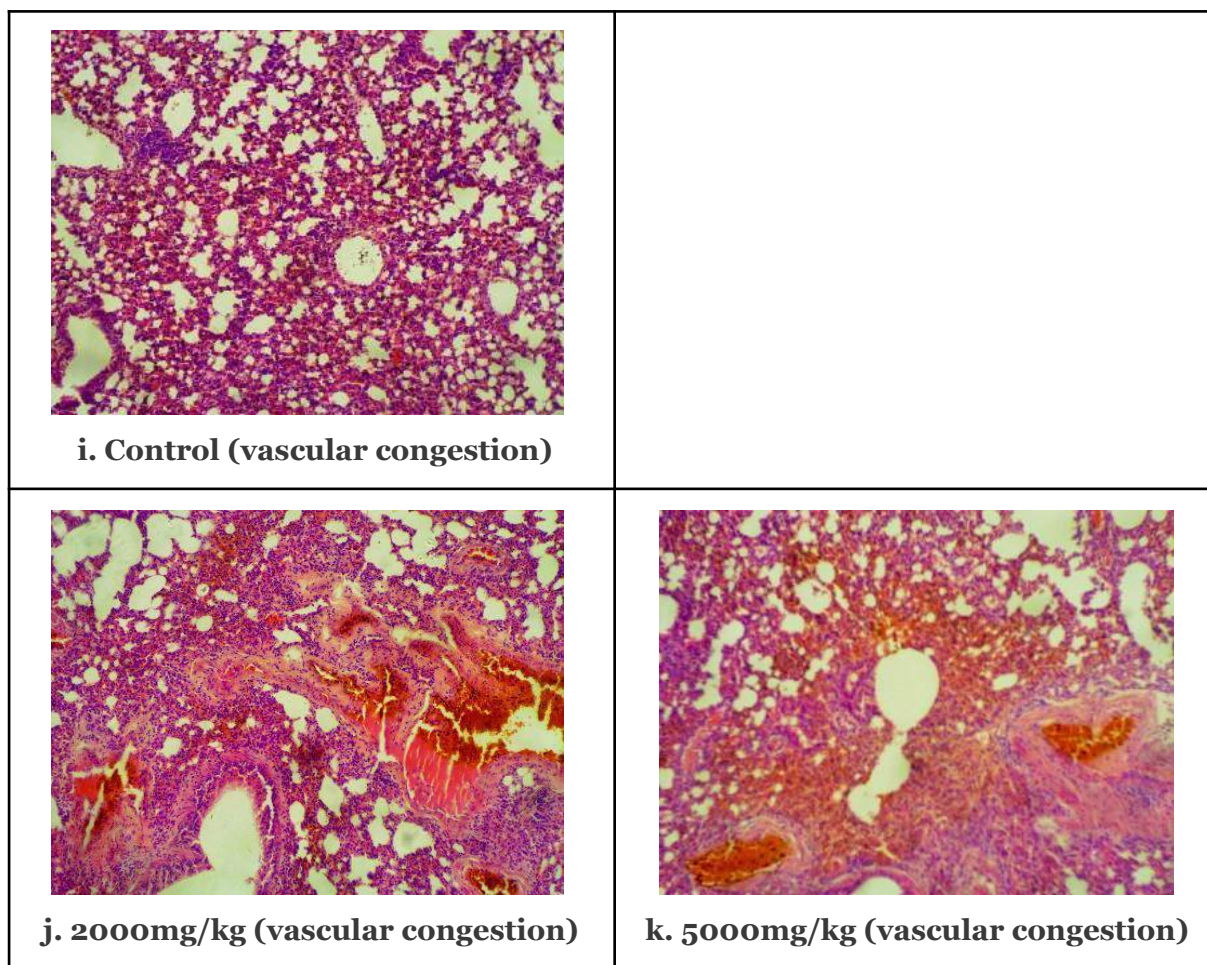


Figure 8: Histologic sections of lung tissue showing some alveolar filled air spaces, the vessels in the walls of the alveoli are distended and the capillaries are congested with aggregates of many red blood cells. The lung sections for untreated and those treated with 300-5000mg/kg of ethanolic extract of *Picralima nitida* had mild to severe vascular congestion

IV. DISCUSSION

The phytochemical analysis showed the presence of alkaloids, tannins, phenols, saponin, flavonoids, and terpenoids which indicated that these plants contain important plant antioxidants and the plant may thus have free radical scavenging activities. Our result is similar with previous research by Nkere and Iroegbu who reported the presence of alkaloids, tannins, saponins, flavonoids, terpenoids, and steroids in *Picralima nitida* [11]. The LD₅₀ value obtained from our acute toxicity study indicates that *Picralima nitida* is safe. Our results of the LD₅₀ ($\leq 2000\text{mg/kg}$) is similar to the value reported by Koffi *et al* who estimated the LD₅₀ values for the acute oral and intraperitoneal toxicity studies for *Picralima nitida* to be 3000 mg/kg [12].

The absence of histological alterations in the heart histology of both untreated and treated groups at low (300mg/kg) to the highest dose (5000mg/kg) indicate that aqueous extract is safe on the heart even at the highest dose. However, the presence of some histologic changes in the liver, kidneys, lungs of the untreated (control) and treated groups that received aqueous extract of *Picralima nitida* indicate toxic adverse effects which may not be induced by the *Picralima nitida* because the same histological injuries were also seen in the control. Hence, the cause of the adverse effects is unknown.

The histologic sections of the 80% ethanolic extract of *Picralima nitida* treated animals showed some histopathology in the heart, liver, kidney, and lungs of the animals that received mid

dose (2000mg/kg), and high dose (5000mg/kg). This could be a spontaneous occurrence because similar injuries were also seen in the untreated group (control) thus the cause of the histopathology is unclear.

V. CONCLUSION

The phytochemical screening revealed that *Picalima nitida* contains important antioxidants and other phytochemicals with various health benefits. While the acute toxicity assessment of the aqueous and 80% ethanolic extracts of *Picalima nitida* indicate that *Picalima nitida* is safe for oral consumption and subsequent therapeutic activities.

Conflict of Interests: The authors declare no conflict of interest.

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Effectiveness of Radiotherapy in Combination with Adjuvant Selective Intra-Arterial Chemotherapy in the Treatment of Malignant Neoplasms of the Orbit

Maletskyy A.P.

INTRODUCTION

Malignant tumors of the orbit make 41-45.9% [4, 5] and they represent a threat to both the organ of vision and the patient's life. Thus, according to a number of authors [5], malignant tumor recurrence of the orbit within 5 years was observed in 36 out of 56 observed patients, ie. the tumor recurrence was observed in 64.3% of patients, who died in the following years. As is evident from the above data, the results of treatment of malignant tumors of the orbit are not satisfactory. In the analyzed group of 56 patients with malignant tumors, the 5-year survival was only 36%.

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Effectiveness of Radiotherapy in Combination with Adjuvant Selective Intra-Arterial Chemotherapy in the Treatment of Malignant Neoplasms of the Orbit

Maletskyy A.P.

Author: Institute of Ophthalmic Diseases and Tissue Therapy, V.P. Filatov of NAMS of Ukraine, Odessa, Ukraine.

selective intra arterial chemotherapy (cisplatin) in the treatment of malignant orbital tumors.

I. INTRODUCTION

Malignant tumors of the orbit make 41-45.9% [4, 5] and they represent a threat to both the organ of vision and the patient's life. Thus, according to a number of authors [5], malignant tumor recurrence of the orbit within 5 years was observed in 36 out of 56 observed patients, ie. the tumor recurrence was observed in 64.3% of patients, who died in the following years. As is evident from the above data, the results of treatment of malignant tumors of the orbit are not satisfactory. In the analyzed group of 56 patients with malignant tumors, the 5-year survival was only 36%.

The basic method of treatment of malignant tumors of the orbit is surgical treatment consisting of removal of the tumor, possibly with subsequent radiotherapy and chemotherapy [1, 3]. However, surgical treatment of malignant tumors of the orbit leads to anatomical and functional disorders. Independent use of radiation therapy does not lead to the desired result. It seems to us that the increase in the efficiency of radiation therapy and minimization of negative consequences of systemic chemotherapy can be achieved by including selective intraarterial chemotherapy in the complex treatment of malignant orbital tumors, which allows us to create a higher concentration of the drug in the tumor itself and, consequently, increases its sensitivity to radiation therapy [2, 6].

Aim: of this research is to study the effectiveness of radiation therapy in combination with adjuvant

II. MATERIAL AND METHODS

Treatment was analyzed in 21 patients (age 45.6 ± 2.9 years, men - 9 cases, women - 12). By the nature of treatment the patients were divided into two groups. The first group consisted of 7 patients, who at the first stage had undergone orbital tumor removal with the following histological examination (orbital melanoma - 2, eyelid and orbit melanoma - 2, a mixed tumor of the lacrimal gland - 1, histiocytoma and angiosarcoma of the orbit - 2). Observation period (12-64 months). The second group consisted of 14 patients who had previously had a tumor biopsy followed by histological examination (orbital melanoma - 2, eyelid and orbit melanoma - 6, cylinderoma - 2, osteoclastoma - 1, lacrimal gland adenocarcinoma - 1, rhabdomyosarcoma - 1 and angiosarcoma - 1), then followed by combined treatment, which consisted of administration of cisplatin into the maxillary artery and parallel radiation therapy focused on the tumor growth area. It should be noted that in all 6 cases, the flat cell carcinoma affected the maxillary sinus and the lattice bone, and in one case - spread into the middle cranial pit. The essence of the intra-arterial chemotherapy technique was that the catheter is retrogradely inserted through the external temporal artery (to a depth of 2-3 cm) to the mouth of the maxillary artery (Fig. 1). The position of the catheter was controlled during the operation using a 0.5% solution of methylenum coeruleum, which after being inserted into the catheter (0.5-0.7 ml), stained the tissue area corresponding to the blood supply area of the maxillary artery.



Fig. 1. 1: Catheter is put into temporal artery, 2 - control of it's position with the help of 0,5% solution methylenum coeruleum

In addition, control angiography was performed prior to cisplatin infusion to determine the correct position of the catheter (Fig. 2).

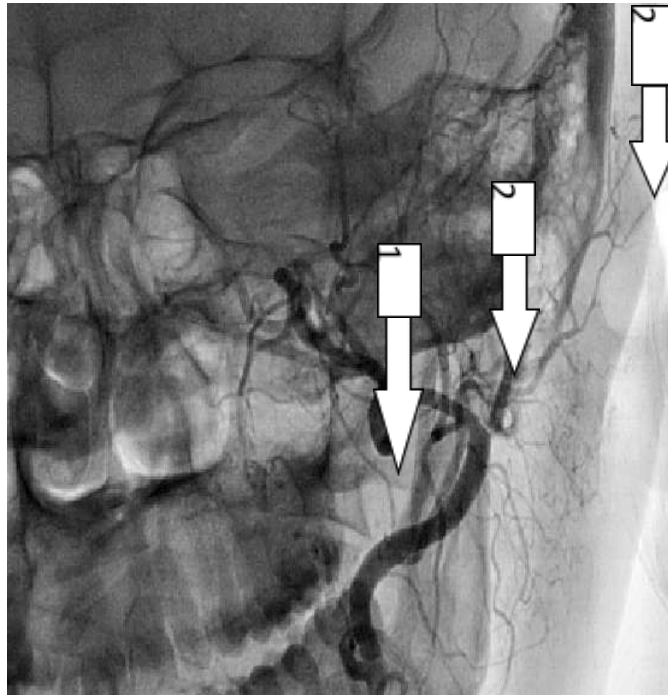


Fig. 2: Control angiography (1 - a. maxillaries, 2 - catheter in a. temporalis superficialis)

After that, using a syringe-dispenser, cisplatin is injected at 20-50 mg for 3-7 days (total dose 118.8 + 43.8 mg) (Fig. 3).



Fig. 3: Portable metering device for cisplatin introduction

Telegamma Therapy was carried out in both groups of patients starting from the second day after the beginning of chemotherapy (total dose 45.6 + 14.9 Gy). Evaluation of the treatment results in the first group of patients was carried out according to the presence or absence of relapses. In the second group, treatment was assessed according to the following criteria: early (20-25 days after the end of telegamma therapy) - no tumor regression, partial or complete regression; in the long term (from 4 months and more) - the presence of tumor regression (partial or complete). The observation period is from 3 to 60 months.

III. RESULTS

In 6 out of 7 patients of the first group, tumor recurrence was absent (follow-up period 3-60 months), and in one case, recurrence of upper eyelid carcinoma was marked after 12 months. In patients of the second group, in all cases, a patient-

ts of the second group, in all cases, a positive result of treatment was achieved upon completion of radiation therapy. In six patients (4 cases of cancer, osteoclastoma -1 and 1 - rhabdomyosarcoma), almost complete tumor resorption was marked upon completion of the course of chemotherapy and radiotherapy. Eight patients (adenocarcinoma of the lacrimal gland - 1, cylindroma-2, melanoma of the orbit-2, cancer-2 and angiosarcoma-1) had a partial result during the treatment period, however, at the control examination after 3-4 months and subsequent months (up to 6 months) in 11 patients, complete tumor resorption was achieved, and in one case - long-term growth of orbital angiosarcoma. During the course of the treatment, it was found that the activity of tumor resorption depends on the total dose of the chemotherapy drug and the cellular structure of the tumor. A clinical example is presented as an example (photo 1).



1 - Before treatment



2 - After treatment (in 24 days)

Fig. 4: Patient N., 16 years old, rhabdomyosarcoma of the left orbit

It should be noted that in 14 patients it was possible to maintain visual acuity from 0.1 to 1.0, and in one case with rhabdomyosarcoma of the orbit, it was increased from 0.17 to 0.7. One patient with orbital rhabdomyosarcoma died after 3.5 months. In the course of treatment, a suspicion of lungs metastasis was noted, which was subsequently confirmed.

IV. CONCLUSION

The analysis of preliminary clinical results showed that intra-arterial administration of cisplatin significantly increases the sensitivity of tumor cells to radiotherapy, and, therefore, makes it

possible to activate the intensity of tumor resorption and achieve a higher clinical and functional result. However, the analysis carried out on a small number of patients and in a short follow-up period does not allow us to make final conclusions about rational chemotherapy regimens and the effectiveness of treatment in relation to tumor recurrence and the prognosis of the patient's life, therefore, research in this direction will be continued.

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Digoxin was almost Abandoned in HFrEF Therapy. Is that Entirely Justified?

Vjeran Nikolić Heitzler

INTRODUCTION

Digoxin is a positive inotropic agent, the only one suitable for chronic oral administration in patients with systolic heart failure (HFrEF <45-50%) with or without atrial fibrillation. Neuro-humoral properties are also significant by suppressing excessive activity of the sympathetic and renin-aldosterone systems. Numerous studies confirm that it achieves exceptional hemodynamic effects by increasing EJECTION FRACTION (EF), cardiac index by reducing pulmonary capillary pressure. It slows down the heart and neutrally affects blood pressure. Therefore, unlike beta-blockers and ACEs / ARBs, it can be safely used in patients with lower blood pressure. Digoxin is also associated with an improvement in renal function, estimated to increase glomerular filtration rate by 20%. Therefore, unlike renin-angiotensin-aldosterone inhibitors, it can be administered to patients with borderline renal function without the risk of further renal impairment (1,2,3,4,5)

Keywords: digoxin, Heart failure with reduced ejection fraction (HFrEF), mortality, hospital readmission.

Classification: NLMC CODE: QV 745, WG 200

Language: English



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Digoxin was almost Abandoned in HFrEF Therapy. Is that Entirely Justified?

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Keywords: digoxin, Heart failure with reduced ejection fraction (HFrEF), mortality, hospital readmission.

Author: Poliklinic “Ivo Drinković”, Zagreb, Šulekova 5, HR. 10000 Zagreb, Croatia.

I. INTRODUCTION

Digoxin is a positive inotropic agent, the only one suitable for chronic oral administration in patients with systolic heart failure (HFrEF <45-50%) with or without atrial fibrillation. Neuro-humoral properties are also significant by suppressing excessive activity of the sympathetic and renin-aldosterone systems. Numerous studies confirm that it achieves exceptional hemodynamic effects by increasing EJECTION FRACTION (EF), cardiac index by reducing pulmonary capillary pressure. It slows down the heart and neutrally affects blood pressure. Therefore, unlike beta-blockers and ACEs / ARBs, it can be safely used in patients with lower blood pressure. Digoxin is also associated with an improvement in renal function, estimated to increase glomerular filtration rate by 20%. Therefore, unlike renin-angiotensin-aldosterone inhibitors, it can be administered to patients with borderline renal function without the risk of further renal impairment (1,2,3,4,5)

II. DISCUSSION

It was the drug of first choice for many years until a 1997 DIG study showed that digoxin did not reduce mortality in that population (NYHA III-IV with LVEF≤40% or NYHA II with LVEF≤ 30% with or without atrial fibrillation), but contributes to the reduction of symptoms and the frequency of hospitalizations. The DIG study may complain that beta-blockers and aldosterone antagonists

were not used in the treatment of heart failure at that time, and relatively high doses of digoxin were prescribed, which are not common today. Negative remarks on DIG study /1997/.

1. HF therapy without beta-blockers and aldosterone antagonist
2. High digoxin doses 0.25 mg
3. Distribution of NYHA class I = 13.3%
II = 53.3%
III = 30.7%
IV = 2.2%

small percentage representation of more severe HF cases.

1. 44,1% of the patients assigned to digoxin were on digoxin before study entry.
2. 44,6% of the patients allocated to placebo were previously on stable, chronic digoxin therapy without a wash-out period (6,7)

In the last twenty years alone, the use of digoxin in HFrEF therapy has dropped by rather than two thirds. According to a US study (GWTH-HF) on 250,000 patients with HFrEF, the frequency of digoxin use in discharge therapy decreased from 33.1% of patients in 2005 to 10.7% in 2014. (8).

Current European IIb and American Guideline IIa recommend digoxin in patients with HFrEF who have persistent symptoms despite optimal therapy, in order to reduce the frequency of hospitalizations. Small doses equivalent to a serum concentration <0.9 ng / ml are recommended when digoxin is administered.

In studies with stable HFrEF when digoxin therapy was discontinued, symptoms worsened, exercise tolerance decreased, and a decrease in EF was recorded. In extremely severe cases of HFrEF, the introduction of digoxin was able to remove

mechanical circulatory support and intravenous inotropic drugs (9,10,11,12).

Heart failure therapy has changed greatly in the last twenty years or so. In addition to modern therapy: angiotensin receptor neprilysin inhibitor (ARNI) sacubitril/valsartan is now preferred compared to ACEI/ARBs and sodium-glucose cotransporter-2 inhibitors (SGLT2) are now an option for patients with or without diabetes after ARNI/ACEI/ARB and BB treatment has started. Finally, mechanical support and heart transplantation are today a possibility in advanced stage of the disease, if every other therapy fails(14).

III. CONCLUSION

The clinician faces the dilemma of relying on the quality of data arising from clinical trials conducted more than two decades ago and before modern heart failure therapy was available or on evidence from mainly observational studies .

Digoxin probably still has an excuse in patients with severe advanced systolic dysfunction who are unable to tolerate high doses of drugs due to limits in blood pressure, renal function. Digoxin should be used to reduce repeated hospitalizations and today's systolic heart failure patient is on average 10 years older than in the DIG study and a daily dose of 0.10 may be appropriate for a greater number of patients(13).The pharmaceutical industry has no interest in a new DIG study no matter how previous we may blame a number of shortcomings since digoxin it is a very cheap drug (12).

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Antitumor Effects of Zoledronic Acid under Hypoxia

*Erik Henke, Bettina Brendel, Herbert Stolz, Elke Butt-Dörje, Alma Zerneck-Madsen, Andreas Beilhack
& Prof Dr. Friedrich Schardt*

Universitätsklinikum Würzburg

ABSTRACT

Bisphosphonates are widely used in the clinic for the treatment of osteoporosis, osteogenesis imperfecta, fibrous dysplasia and of various malignancies. In cancer treatment they are mainly used palliatively to reduce loss of bone density as a result of metastasis. In addition, several reports also claim a direct effect on the tumor cells and improved survival under bisphosphonate treatment. However, the anti-tumor effect of bisphosphonates remains controversial.

In this study we explored the glycolysis blocking properties of the bisphosphonate zoledronic acid in leukemia and breast cancer cells. Although, zoledronic acid had little effect at normoxic conditions, it significantly inhibited lactate production at reduced oxygen levels. Under these hypoxic conditions, that resemble the oxygenation levels in many tumors, zoledronic acid was also of significantly higher toxicity to the tumor cells. Moreover, we show that it strongly increased sensitivity to chemotherapy.

These results support the Warburg hypothesis and encourage further testing in vivo to explore a potentially beneficial effect of zoledronic acid on the response to chemotherapy.

Keywords: bisphosphonates, hypoxia, tumor cells, anaerobic glycolysis, bone metastasis.

Classification: NLMC CODE: WA 730

Language: English



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Antitumor Effects of Zoledronic Acid under Hypoxia

Erik Henke^α, Bettina Brendel^σ, Herbert Stolz^ρ, Elke Butt-Dörje[¥], Alma Zerneck-Madsen[§],
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ABSTRACT

Bisphosphonates are widely used in the clinic for the treatment of osteoporosis, osteogenesis imperfecta, fibrous dysplasia and of various malignancies. In cancer treatment they are mainly used palliatively to reduce loss of bone density as a result of metastasis. In addition, several reports also claim a direct effect on the tumor cells and improved survival under bisphosphonate treatment. However, the anti-tumor effect of bisphosphonates remains controversial.

In this study we explored the glycolysis blocking properties of the bisphosphonate zoledronic acid in leukemia and breast cancer cells. Although, zoledronic acid had little effect at normoxic conditions, it significantly inhibited lactate production at reduced oxygen levels. Under these hypoxic conditions, that resemble the oxygenation levels in many tumors, zoledronic acid was also of significantly higher toxicity to the tumor cells. Moreover, we show that it strongly increased sensitivity to chemotherapy.

These results support the Warburg hypothesis and encourage further testing in vivo to explore a potentially beneficial effect of zoledronic acid on the response to chemotherapy.

Keywords: bisphosphonates, hypoxia, tumor cells, anaerobic glycolysis, bone metastasis.

Author α: Institute of Anatomy and Cell Biology, Universität Würzburg.

σ ρ: Central Laboratory, Universitätsklinikum Würzburg.

¥ §: Institute of Experimental Biomedicine, Universitätsklinikum Würzburg.

x: Department of Internal Medicine II, Center for Experimental Molecular Medicine, Universitätsklinikum Würzburg.

v: Betriebsärztliche Untersuchungsstelle, Universitätsklinikum Würzburg.

I. INTRODUCTION

Bisphosphonates are stable synthetic analogues of pyrophosphate that are resistant to metabolic hydrolysis and applied as inhibitors of osteoclasts against bone resorption. In this function they are applied as palliative treatment in patients with manifested bone metastases in the context of breast cancer (reviewed in [1, 2]). However, a potential effect in preventing bone metastases or as adjuvant treatment to prevent therapy induced bone loss has been discussed [3-5].

Osteoclasts disassemble and digest the composite of hydrated protein and mineral in bones at a molecular level by secreting acid phosphates and collagenases [6]. They are found in pits, so called resorption bays or Howship's lacunae, and in niches of the bone marrow, locations that are characterized by a reduced oxygen partial pressure (pO₂) [7]. These particular micro-environments resemble niches for hematopoietic stem cells.

Because of their hypoxic environment, the metabolism of osteoclasts and malignant tumor cells depend on an increased rate of glycolysis, which is sustained by a very high glucose import rate as the glucose molecule has the highest percentage of oxygen comparable to fat and proteins [8-11]. Therefore, substances that inhibit glycolysis are prone to interfere with the metabolism of these cells and appear particularly attractive for supporting cancer treatment. This holds especially true for the targeting of cancer stem cells (CSCs) or tumor initiating cells (TICs) in certain cancer types. Especially CSCs/TICs breast, colon and hepatocellular cancer rely

heavily on glycolysis [12-16], whereas in glioblastoma, lung and pancreatic cancer CSCs/TICs revert more to oxidative phosphorylation for ATP-synthesis [17-19]. The CTCs seem to depend on their respective metabolic program to maintain their stem-like properties. Beside their ability to initiate and repopulate tumors, CSCs are characterized by chemoresistance. Therefore, we asked whether targeting glycolysis in types of cancers, in which CSCs and TICs depend on this metabolic process would make them vulnerable, increase treatment sensitivity and decrease metastasis.

As bisphosphonates can interfere with glycolysis, we investigated whether their application could interfere with glycolysis in cancer under hypoxic conditions. Here we report that zoledronic acid, a potent bisphosphonate, reduced lactate production in leukemia and breast cancer cell lines under hypoxic conditions. Importantly, zoledronic acid potentiated cytotoxic effects of chemotherapeutic agents against breast cancer under hypoxic conditions.

II. RESULTS

To test our hypothesis that bisphosphonates can interfere with glycolysis, a metabolic pathway activated under hypoxic conditions and necessary for stem cell maintenance, we first examined the effect of zoledronic acid on cell lines of acute and chronic myeloid leukemia (AML, CML). Leukemia cells generally express high levels of *bona fide* stem cell markers [20]. Inhibition of glycolysis activity should affect levels of lactate produced by the cells. Indeed, lactate concentration in the supernatant of cells treated with zoledronic acid at various concentration (1 μ M, 10 μ M and 100 μ M) were significantly lower than in untreated cells (**Figure 1**). However, we observed this effect only under cell culture conditions of reduced oxygen levels at or below 2% O₂ (**Figure 1E-H**). Under normoxia (20% O₂) overall lactate production was lower and not affected by treatment with zoledronic acid (**Figure 1A-D**). Within the four tested lines, THP-1 cells produced by far the least amount of lactate and were the only line showing inconclusive response to zoledronate-treatment. THP-1 cells are a well-differentiated line that can

be readily differentiated [21]. We next tested the response of breast cancer lines to zoledronate-treatment, given the reliance of breast cancer CTCs on glycolysis. Again, a solid effect of zoledronic acid on lactate production was observed in three of the four tested lines at reduced oxygen levels (**Figure 2A**). At normoxia, zoledronate-treatment again had no significant effect on lactate production (**Figure 2B**). The breast cancer lines required higher zoledronate concentrations than the leukemia cells to reduce lactate levels.

To determine effects of zoledronic acid on viability and proliferation, the three sensitive breast cancer cell lines were treated for 72h with a range of concentrations of the drug. All three lines were significantly more sensitive under hypoxic conditions (**Figure 3**).

CTCs are characterized by chemoresistance and contribute significantly to the tumors ability to sustain and rebound after cytotoxic treatment. Sensitivity of breast cancer lines to the microtubule-stabilizing drug paclitaxel was tested in the presence of 1 μ M zoledronate at normoxic and hypoxic conditions. Cultivation at low oxygen levels significantly reduced the cells to the cytotoxic drug (**Figure 4A-B**). Co-treatment with zoledronic acid re-sensitized the cells as the EC₅₀-levels were reduced to values similarly to those found for cells cultivated at normoxia.

III. DISCUSSION

Zoledronic acid can block the phosphorylation from glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate. This reaction proceeds through a thioester intermediate, which allows the oxidation of glyceraldehyde to be complied to 3-phosphoglycerate. Cysteine reacts normally with the aldehyde group of the substrate, forming a hemithioacetal and takes place with the transfer of a hydride ion to NAD. This reaction is facilitated by the transfer of a proton to the imidazole ring of histidine. Zoledronic acid can attach to the thioester instead of an orthophosphate and thereby stops the continuation of the anaerobic glycolysis. The following part of the glycolysis would gain a ΔG

value of -43.9 . However, the anaerobic glycolysis can proceed only if the ΔG values of all reactions are negative. In contrast, the first part of anaerobic glycolysis has a positive ΔG of 5.7 ($-30.9 + 36.6$). Additionally, NAD from the reduction of pyruvate to lactate is not available for this process of the glycolysis in cytosol of the cell.

In this study, we examined the glycolysis blocking properties of zoledronic acid in cancer. Accordingly, we observed reduced lactate production in different cancer cell lines upon zoledronate treatment. Notably, this effect happened only under hypoxic conditions, when anaerobic glycolysis gains relevance to sustain the high metabolic demands of cancer cells. As a consequence, the breakdown of pyruvate to lactate was interrupted. We observed this effect in less differentiated cancer cells, whereas more differentiated cancer cell lines such as THP-1 or MCF-7 were less affected. These results emphasize that less differentiated cancer cells, including CTCs and TICs in certain cancer types depend more on glycolysis, which makes them more vulnerable to disruption of glycolysis. Indeed, when we combined zoledronate with paclitaxel as a chemotherapeutic agent to increase the cellular stress level for CTC-like breast cancer cells, they became markedly more vulnerable upon adding the bisphosphonate. These results are encouraging to be tested in vivo, as metastases to hypoxic bone niches and ensuing chemoresistance pose a therapeutic hurdle to effectively treat breast cancer patients.

IV. MATERIAL AND METHODS

Chemicals were acquired from standard commercial suppliers (Sigma Aldrich, Merck). Zoledronic acid was acquired as a 0.8 mg/mL solution from Denk Pharma (Munich, Germany) and diluted from this stock accordingly. Cell culture media and supplements were purchased from Thermo Fisher (Germany).

4.1 Cell lines

All cell lines were acquired from ATCC. Breast cancer cell lines (MDA-MB231, MDA-MB435s, MDA-MB-468, MCF-7 and SkBr-3) were maintained in DMEM supplemented with 10%

FBS and penicillin/streptomycin. The leukemia cell lines (MO7, HL-60, THP-1 and K-562) were maintained in RPMI 1640 media supplemented with 10% FBS and penicillin/streptomycin.

4.2 Lactate measurement

Adherent growing cells were seeded at 2×10^4 cells in 24-well MWD dishes in $500 \mu\text{L}$ in lactate and pyruvate free media. To attach, cells were maintained at normoxia for 24 h. Then one set of cells were transferred to a hypoxia incubator set at 2% O_2 , while a second set was maintained at normoxia. Cells were allowed to adjust to the conditions for 24h, before they were supplied with fresh media containing the appropriate amount of zoledronic acid. Media was collected after 24h and frozen at -80°C .

Cells growing in suspension were treated analogously. For media exchange and to harvest growth media at the end of the experiment cells were separated by centrifugation ($300 \times g$ for 5 min).

Lactate concentration and LDH activity was determined vs. pyruvate levels using standard conditions given by the manufacturer on a Cobas 8000 modular analyzer (Roche, Mannheim, Germany):

4.3 Assessment of cell viability

In cell toxicity studies each concentration was tested in a 6-fold replicate. Cells were incubated with the therapeutics for 72 h before media was removed and cells stored at -80°C until further quantification using the CyQuant assay kit (ThermoFisher, Germany) according to the manufacturer's instructions.

4.4 Cytotoxicity of zoledronic acid

Adherent growing cells were seeded at 1×10^3 cells in 96-well MWD dishes in $200 \mu\text{L}$ in standard media. To attach, cells were maintained at normoxia for 24 h. Then one set of cells were transferred to a hypoxia incubator set at 2% O_2 , while a second set was maintained at normoxia. Cells were allowed to adjust to the conditions for 24h, before they were supplied with fresh media supplemented with zoledronic acid. Zoledronic

acid was applied at 9 different concentrations at a range from 4.6 nM to 30 μ M. Cells were re-incubated at the respective O₂-levels (2% or 20%) and cell viability was assessed 72 h later.

For each tested cell line (MDA-MB-231 and MDA-MB468) four 96-well MWDs were prepared, seeding cells at 1×10^3 cells in 200 μ L using standard media. In two of those plates media was supplemented with 1 μ M zoledronic acid. Two plates, one with and one without added zoledronic acid, were transferred to a hypoxia incubator set at 2% O₂, the two other plates were maintained at normoxia. Cells were allowed to adjust to the conditions for 24h, before they were supplied with fresh media supplemented with paclitaxel. Paclitaxel was applied at 9 different concentrations at a range from 0.46 nM to 3 μ M. Cells were re-incubated at the respective O₂-levels (2% or 20%) and cell viability was assessed 72 h later.

4.5 Statistical Analysis

All statistical analysis was done using the Prism5 Software (GraphPad, LaJolla, CA). Differences between two groups were analyzed using an unpaired, two-tailed Student's T-test. In parallel the samples were tested for significant variation of variance, and if necessary, a Welch correction was included in the statistical analysis.

All authors reviewed this article.

The authors declare no competing interest.

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Figure Legends

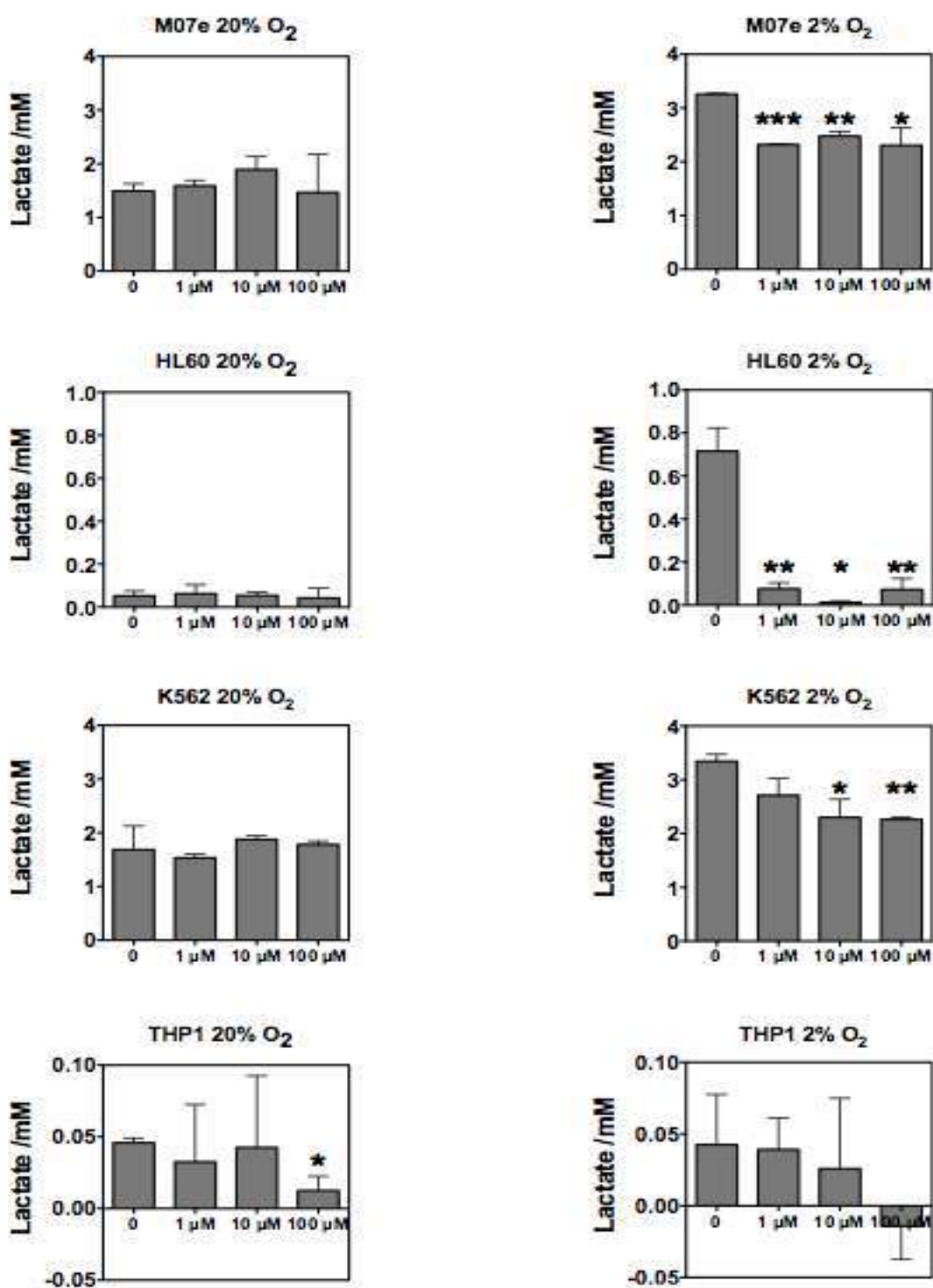


Figure 1: Zoledronic acid treatment increases lactate production in myeloid leukemia cell lines under hypoxic conditions

(A-D) Lactate concentration in the supernatant of four myeloid leukemia cell lines cultivated under normoxia (20 % O₂) and treated with increasing concentrations of zoledronic acid.

(E-F) Lactate concentration in the supernatant of the same four myeloid leukemia cell lines cultivated under hypoxia (2 % O₂) and treated with increasing concentrations of zoledronic acid.

Error bars: +/- SEM, *: P < 0.05, **: P < 0.01, ***: P < 0.001.

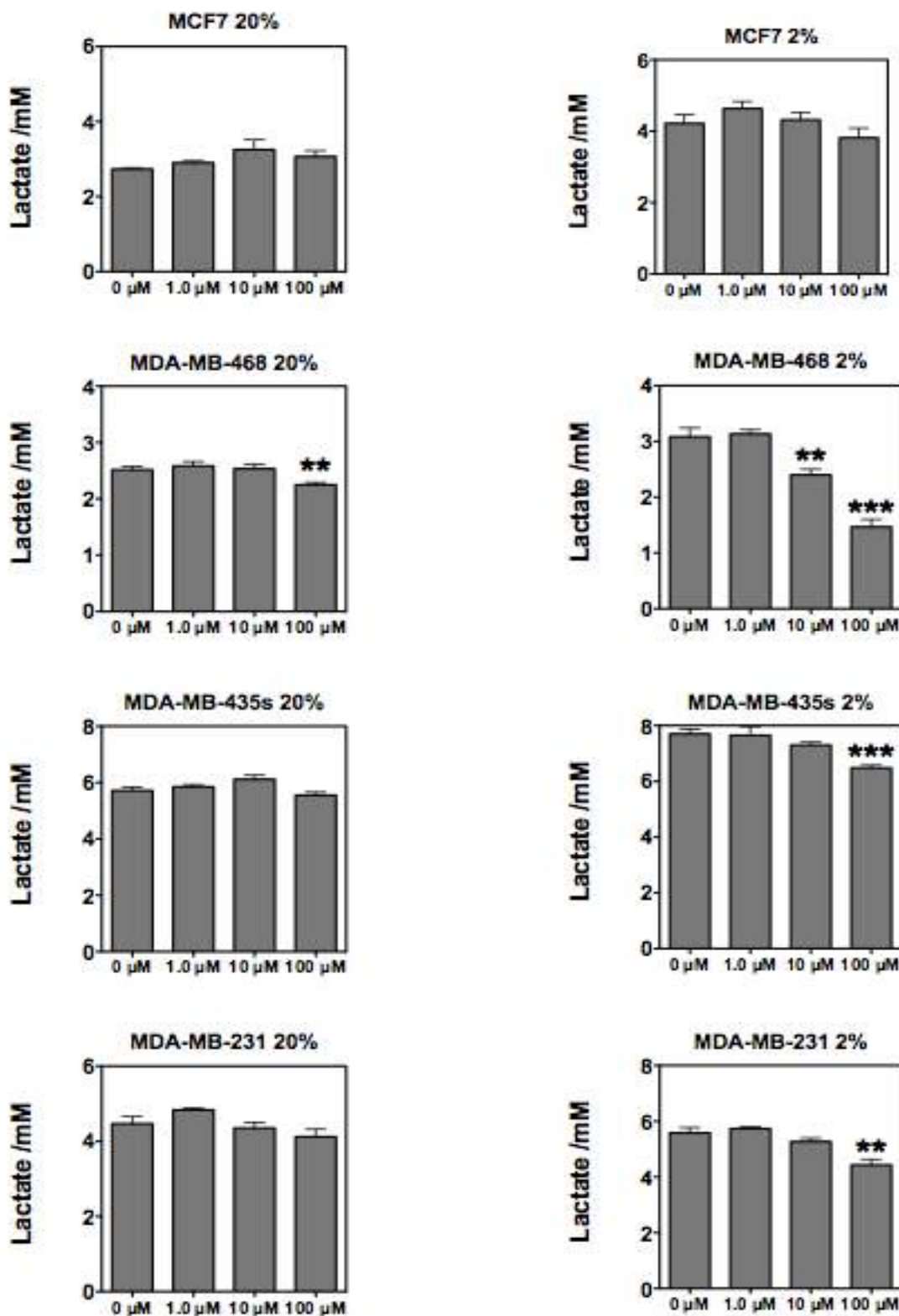


Figure 2: Effect of zoledronic acid on lactate production in breast cancer cell lines.

(A) Lactate concentration in the supernatant of four breast cancer cell lines cultivated under hypoxia (2 % O₂) and treated with increasing concentrations of zoledronic acid.

(B) Lactate concentration in the supernatant of the same four breast cancer cell lines cultivated under normoxia (20 % O₂) and treated with increasing concentrations of zoledronic acid.

Error bars: +/- SEM, *: P < 0.05, **: P < 0.01, ***: P < 0.001.

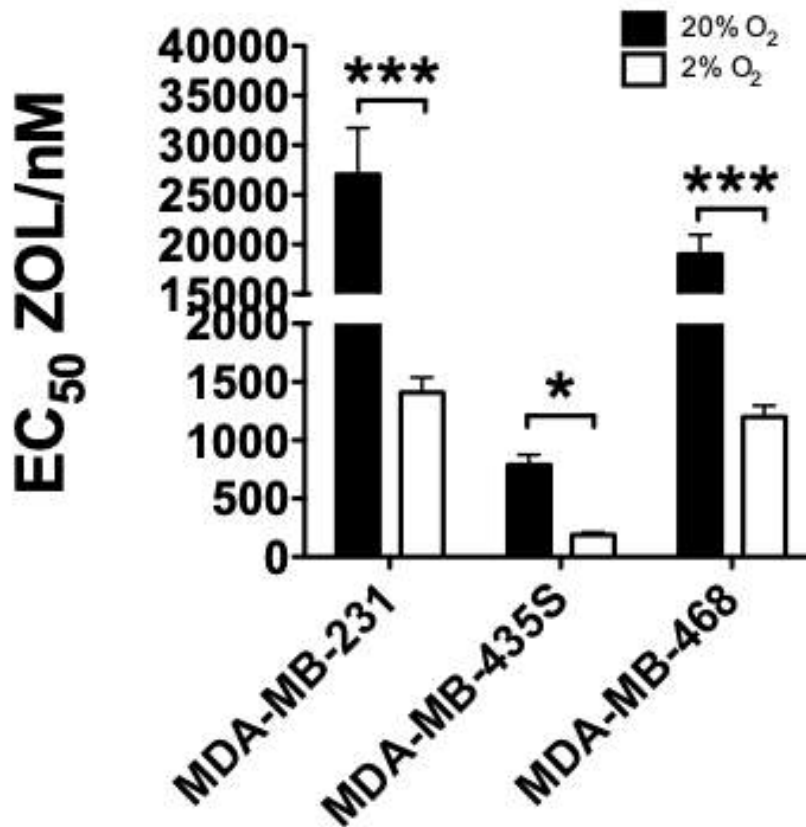


Figure 3: Cell toxicity of zoledronic acid on breast cancer cell lines

EC₅₀-values of zoledronic acid in three breast cancer cell lines under normoxia (20 % O₂) and hypoxia (2 % O₂) Error bars: +/- SEM, *: P < 0.05, **: P < 0.01, ***: P < 0.001.

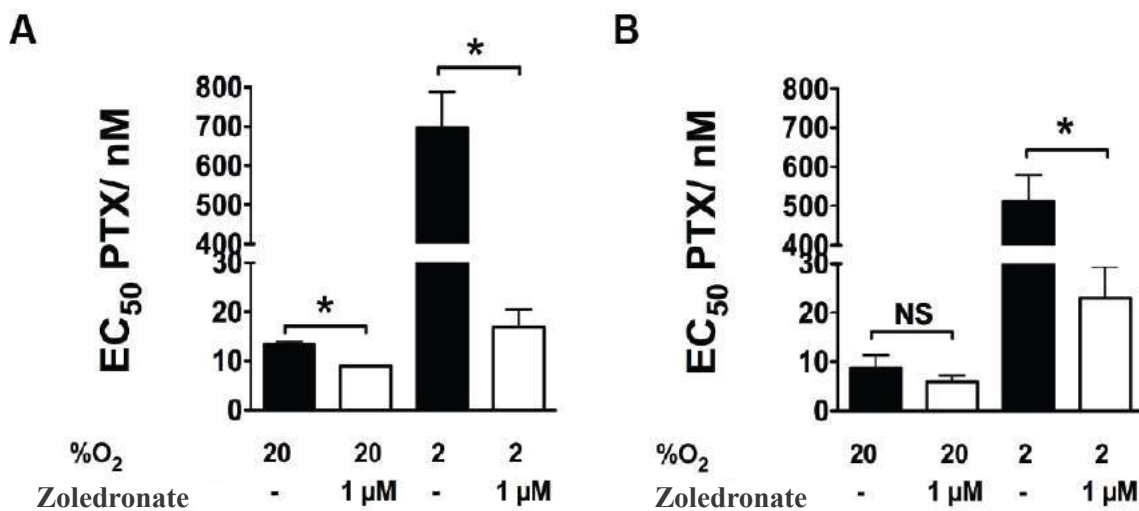


Figure 4: Cell toxicity of paclitaxel (PTX) on breast cancer cell lines pre-treated with zoledronic acid

(A) EC₅₀-values of PTX in MDA-MB-231 breast cancer cells in dependence of pre-treatment with 1 μM zoledronic acid and of oxygenation status (20 % O₂ vs. 2 % O₂).

(B) EC₅₀-values of PTX in MDA-MB-468 breast cancer cells in dependence of pre-treatment with 1 μM zoledronic acid and of oxygenation status (20 % O₂ vs. 2 % O₂).

Error bars: +/- SEM, *: P < 0.05, **: P < 0.01, ***: P < 0.001.

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