PHYTOCHEMICAL COMPOSITION, ANTIBACTERIAL EFFICACY, AND SYNERGISTIC INTERACTIONS OF *CLINOPODIUM NEPETA* SUBS *ASCENDENS* ESSENTIAL OIL IN COMBINATION WITH CONVENTIONAL ANTIBIOTICS

TAIBI, M.[1,2*] – ELBOUZIDI, A.[1,5] – HADDOU, M.[1,2] – BELBACHIR, Y.[3] – BARAICH, A.[4] – LOUKILI, E. H.[5] – YAHYAOUI, M. I.[4] – BENTOUHAMI, N. E.[4] – MOUMNASSI, S.[4] – BELLAOUCHI, R.[4] – ASEHRAOU, A.[4] – CAPANOGLU, E.[6] – ADDI, M.[1] – BOURHIA, M.[7*] – IBENMOUSSA, S.[8] – SALAMATULLAH, A. M.[9] – EL GUERROUJ, B.[1,2] – CHAABANE, K.[1]

[1]*Laboratory of Agricultural Production Improvement, Biotechnology, and Environment (LAPABE), Faculty of Sciences, Mohammed First University, Oujda 60000, Morocco (e-mails: mohamedtaibi9@hotmail.fr, amine.elbouzidi@ump.ac.ma, haddou.mounir27@gmail.com, m.addi@ump.ac.ma, k.chaabane@ump.ac.ma)*

[2]*Center for Oriental Water and Environmental Sciences and Technologies (COSTEE), Mohammed First University, Oujda 60000, Morocco (e-mail: elguerroujb@gmail.com)*

[3]*Physical Chemistry of Natural Resources and Process Team, Laboratory of Applied Chemistry and Environment (LCAE), Department of Chemistry, Faculty of Sciences, Mohammed First University, Oujda, Morocco (e-mail: yousra.belbachir12@gmail.com)*

[4]*Laboratory of Bioresources, Biotechnology, Ethnopharmacology and Health, Faculty of Sciences, Mohammed First University, Boulevard Mohamed VI, B.P. 717, Oujda 60000, Morocco (e-mails: abdellah.baraich@ump.ac.ma, iy.meryem@ump.ac.ma, noureddine.bentouhami@ump.ac.ma, sara.moumnassi@ump.ac.ma, r.bellaouchi@ump.ac.ma, asehraou@yahoo.fr)*

[5]*Euro-Mediterranean University of Fes (UEMF), Fes, Morocco (e-mail: e.loukili@ump.ac.ma)*

[6]*Department of Food Engineering, Faculty of Chemical and Metallurgical Engineering, Istanbul Technical University 34469 Istanbul, Turkey (e-mail: capanogl@itu.edu.tr)*

[7]*Department of Chemistry and Biochemistry, Faculty of Medicine and Pharmacy, Ibn Zohr University, Laayoune 70000, Morocco*

[8]*Laboratory of Therapeutic and Organic Chemistry, Faculty of Pharmacy, University of Montpellier, Montpellier 34000, France*

[9]*Department of Food Science & Nutrition, College of Food and Agricultural Sciences, King Saud University, 11 P.O. Box 2460, Riyadh 11451, Saudi Arabia*

***Corresponding authors**
e-mail: mohamedtaibi9@hotmail.fr, m.bourhia@uiz.ac.ma

(Received 16th Feb 2024; accepted 13th Jun 2024)

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2205_41014114

Abstract. The principal objective of the present study is to evaluate both the chemical composition and antibacterial effectiveness of essential oil of *Clinopodium nepeta* subs *ascendens* (CNEO) against three Gram-positive bacteria (*Staphylococcus aureus*, *Micrococcus luteus*, and *Bacillus subtilis*) and three Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*). Additionally, this research aims to explore potential synergies between CNEO and established antibiotics. The phytochemical composition of CNEO was thoroughly examined via gas chromatography mass spectrometry (GC-MS), revealing neoisomenthol (37.89%), pulegone (20.11%), and dihydrocarvone (19.01%) as the primary bioactive molecules. CNEO exhibited substantial antibacterial activity, with minimum inhibitory concentration (MIC) values ranging from 0.10 against *E. coli* to 1 mg/mL against *K. pneumoniae*. This promising antimicrobial activity, especially noteworthy against antibiotic-resistant bacteria, prompted an evaluation of CNEO's synergistic potential with antibiotics using the Fractional Inhibitory Concentration Index (FICI). The FICI analysis, especially with ampicillin against *P. aeruginosa*, uncovered an optimal synergistic interaction at a FICI of 0.31. Notably, observed reductions in MIC values for CNEO and antibiotics, ranging from 2-7 folds, highlight the heightened efficacy of these combinations. These findings hold significance for developing new approaches to address the increasing challenges of bacterial infections resistant to conventional treatments.
Keywords: *Gram-positive bacteria, Gram-negative bacteria, GC-MS, antibiotic-resistant bacteria, Fractional Inhibitory Concentration Index, synergistic interaction, combined therapies*

Introduction

Antibiotics have marked a major revolution in the treatment of infections since their discovery at the beginning of the 20th century (Gould, 2016). Their ability to specifically target bacterial pathogens has represented a significant advance in medicine, saving countless lives and significantly reducing the morbidity associated with various infectious diseases (Maxson and Mitchell, 2016; Dhingra et al., 2020). These antimicrobial agents act by disrupting the vital processes of bacteria, notably by interfering with the synthesis of their cell wall or blocking the production of essential proteins (Etebu and Arikekpar, 2016). These actions ultimately lead to cell death or inhibition of bacterial growth, thereby controlling or even eliminating infection.

However, despite the undeniable success of antibiotics, their widespread and sometimes inappropriate use has led to the emergence of a major global problem: antibiotic resistance (Clark, 2000). This phenomenon is the result of a number of factors, including the excessive and unregulated use of antibiotics in humans and animals, as well as their presence in the environment (Bengtsson-Palme et al., 2018; Chokshi et al., 2019). Antibiotic resistance compromises the effectiveness of these drugs and poses a serious challenge to global public health, making it more difficult to treat common bacterial infections and increasing the risk of serious complications (French, 2005).

As a result, it has become imperative to find alternative and complementary solutions to conventional antibiotics in order to preserve their long-term efficacy and meet the growing need to treat infections. With this in mind, essential oils, extracted from aromatic plants, have emerged as potential alternatives due to their well-documented antimicrobial properties (Raveau et al., 2020). These natural compounds offer a promising approach to the treatment of infections, particularly in the context of growing antibiotic resistance (Langeveld et al., 2014; Rai et al., 2017).

Certain essential oils have shown significant antibiotic potential, capable of inhibiting bacterial growth and fighting infections (Leghari et al., 2021). Among these, the essential oil of *Clinopodium nepeta*, a perennial plant belonging to the Lamiaceae family, is known for its well-documented antimicrobial properties (Debbabi et al., 2020; Vlachou et al., 2023).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2205_41014114

An innovative approach is to explore the potentiation of conventional antibiotics through the synergistic use of essential oils (Langeveld et al., 2014). This combination can not only improve the efficacy of antibiotics, but also reduce the doses required, thereby limiting the risk of resistance developing (El Atki et al., 2019; Rosato et al., 2020).

The aim of this research work is to determine the precise chemical composition of the essential oil of *Clinopodium nepeta subs ascendens* from the eastern region of Morocco and to assess its potential for potentiating antibiotics, in particular amoxicillin, ampicillin, and erythromycin. This study will allow for the exploration of new therapeutic strategies to combat bacterial infections while minimizing the risk of antibiotic resistance.

Materials and methods

Plant material

The study focused on *Clinopodium nepeta subsp. ascendens,* a species native to the province of Ahfir, in the northeastern region of Morocco. Aerial parts of the plant, including leaves and flowers, were methodically acquired from a local market (harvested in spring 2022) and then shipped to the Faculty of Sciences at the Université Mohammed 1er Oujda. The main aim of this botanical study was to subject them to rigorous taxonomic identification, a crucial step in ensuring accurate categorization of the various plant species under consideration. It should be noted that the collection deliberately included the plant's vegetative and reproductive structures, with the aim of facilitating a thorough taxonomic identification, taking into account both the vegetative morphology and floral characteristics of *Clinopodium nepeta subsp. ascendens* (*Fig. 1*).

Figure 1. *Picture of the species Clinopodium nepeta subsp. ascendens. (Photo retrieved the 14th May 2024, from: http://floredupaysbasque.com/pages/plante-498.html)*

Essential oil extraction

The essential oil was extracted from 100 grams of ground plant material obtained from the aerial parts of *Clinopodium nepeta sp. ascendens*. This procedure used the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2205_41014114

hydrodistillation method with a modified configuration of the Clevenger apparatus. This methodological choice was motivated by the need to preserve the volatile components of the essential oil, in line with the methodological guidelines set out in the following research works (El Guerrouj et al., 2023; Taibi et al., 2024a, b).

GC-MS analysis of essential oil

GC-MS analysis was carried out using a Shimadzu GC system, equipped with an RTX-5 column. Pure helium was used at a flow rate of 3 mL/min. Temperatures were maintained at 250°C to ensure accurate results. Mass spectra were generated with an electron energy of 70 eV, covering a mass range from 40 to 300 m/z. Compound identification was carried out by comparing retention times with authenticated standards and analyzing fragmentation patterns. LabSolutions software was used for data processing, guaranteeing accurate and reliable results (Loukili et al., 2021, 2023; Haddou et al., 2023; Taibi et al., 2023a).

Determination of the MIC

The evaluation of the Minimum Inhibitory Concentration (MIC) of CNEO (*Clinopodium nepeta subs ascendens* essential oil) and antibiotics was carried out using a dilution method in modified medium, in accordance with established protocols (El Bouzidi et al., 2024; Elbouzidi et al., 2022; Taibi et al., 2023b). This dilution method involves preparing a series of dilutions of the essential oil and antibiotics in a specifically modified culture medium, enabling us to assess the minimum concentration required to inhibit bacterial growth. A microbial inoculum of the bacterial strains studied, including *Staphylococcus aureus, Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa, Klebsiella pneumoniae*, and *Micrococcus luteus*, was prepared and standardized in Mueller Hinton broth (MHB). CNEO, was dissolved in dimethylsulfoxide (DMSO), and antibiotics were diluted in MHB to obtain specific concentrations ranges. The results of this evaluation revealed variable responses from different bacterial strains, highlighting the diversity of susceptibility profiles to antimicrobial agents. These data contribute to our understanding of bacterial resistance mechanisms and underline the importance of exploring new therapeutic strategies to deal with the emergence of multiresistant strains. These promising results pave the way for new research to explore the interactions between natural compounds and synthetic antibacterial agents, offering innovative prospects for the development of combined therapies and the fight against antibiotic-resistant bacterial infections.

Synergy with synthetic antibiotics

Evaluation of the synergy between CNEO and synthetic antibiotics used the method described previously in reference (Taibi et al., 2024b). The main objective of this study was to analyze the interactions between essential oils and synthetic antibiotics, in particular amoxicillin, ampicillin, and erythromycin. The preferred method was dilution in the medium, with the mixtures then incubation at 37°C for 24 h, followed by careful observation of bacterial growth. To assess the interaction between the substances, the fractional inhibitory concentration index (FICI) was calculated, using specific formulas adapted to essential oils and antibiotics. The results were analyzed using predefined thresholds (Taibi et al., 2024c): $FICI \leq 0.5$ indicates synergy, $0.5 < FICI \leq 0.75$ partial synergy, $0.75 < FICI \leq 2$ without effect and $FICI > 2$ antagonism. The conclusions of

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2205_41014114

this research provide a more enlightening insight into the complex interactions between these compounds, providing directions for improving antibacterial efficacy (Didry et al., 1993). All the experiments were carried out in triplicate in order to guarantee the reliability and accuracy of the results obtained.

Results and discussion

Chemical composition

The chemical composition of *Clinopodium nepeta subs ascendens* essential oil (CNEO) is revealed through the chromatogram illustrated in *Figure 2*, with the identified compounds listed in *Table 1*.

Numerous studies have examined the essential oil extracted from *C. nepeta* (Debbabi et al., 2020; Beddiar et al., 2021). However, this study represents (to the best of our knowledge) the first exploration of essential oil from the Moroccan subspecies *C. nepeta subsp. ascendens*. GC-MS analysis identified 22 compounds, marked by a notable predominance of oxygenated monoterpenes (92.16%), followed by monoterpenes (4.19%), sesquiterpene hydrocarbons (2.58%) and oxygenated sesquiterpene hydrocarbons (1.08%). This essential oil is particularly characterized by the high presence of neoisomenthol (37.89%), pulegone (20.11%), and dihydrocarvone (19.01%).

These terpene compounds are known for their biological and pharmacological properties. The results obtained differ from those reported by Rodenak-Kladniew et al. (2023), who observed that the essential oil of another subspecies of *C. nepeta* (L.), Kuntze subsp. spruneri, is mainly composed of other major terpene compounds such as menthone (26.6%) and isomenthone (11.7%). They also contrast with the results obtained by Öztürk et al. (2021) for oil extracted from *C. nepeta subsp. glandulosum* (Req.), who identified piperitone oxide (47.8%), limonene (18.6%), and piperitone oxide II (13.6%) as the main components. These variations in the chemical constituents of CNEO essential oil can be attributed to various factors, including extraction methods (Hydrodistillation in our study), geographical origin (Ahfir region, with an altitude of 268 m in our study), climatic conditions (a warm, dry Mediterranean temperate climate (Csa) according to the Köppen-Geiger classification) and harvesting period (Spring in our study) (Cherrat et al., 2014; Al-Mijalli et al., 2022).

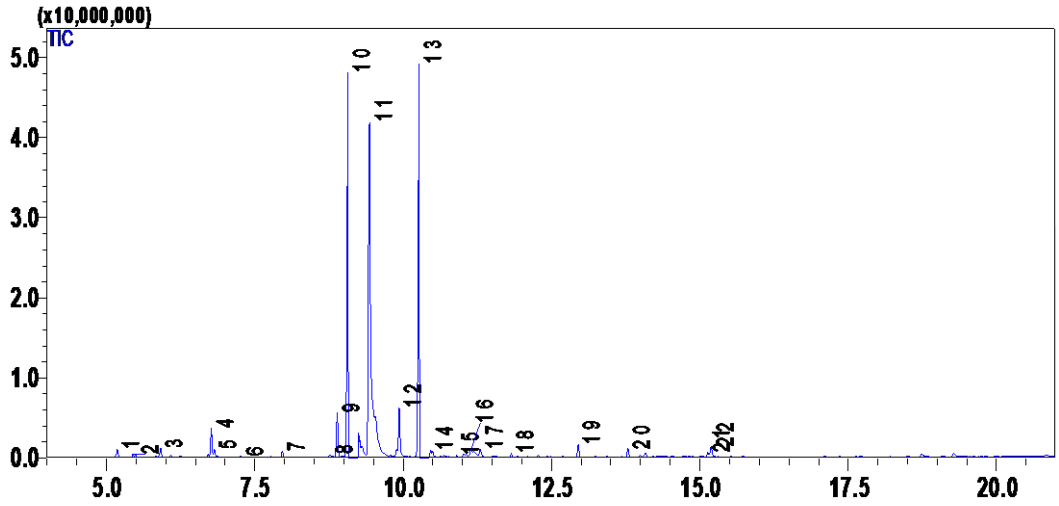

Figure 2. *GC-MS Chromatogram of Clinopodium nepeta subs ascendens essential oil*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2205_41014114

Table 1. *Chemical profile of Clinopodium nepeta subs ascendens essential oil*

N°	Name	RT (min)	% Relative peak area	Type of molecule	Molecular formula
1	a-Pinene	5.184	0.75 ± 0.18	MT	$C_{10}H_{16}$
2	Camphene	5.446	0.11 ± 0.02	MT	$C_{10}H_{16}$
3	b-Pinene	5.912	0.96 ± 0.11	MT	$C_{10}H_{16}$
4	D-Limonene	6.767	2.23 ± 0.08	MT	$C_{10}H_{16}$
5	Eucalyptol	6.819	0.57 ± 0.06	MO	$C_{10}H_{18}O$
6	g-Terpinene	7.265	0.14 ± 0.06	MT	$C_{10}H_{16}$
7	Linalool	7.964	1.98 ± 0.09	MO	$C_{10}H_{18}O$
8	Camphor	8.767	0.41 ± 0.13	MO	$C_{10}H_{18}O$
9	D-menthone	8.885	3.88 ± 0.37	MO	$C_{10}H_{18}O$
10	Dihydrocarvone	9.064	19.01 ± 0.08	MO	$C_{10}H_{16}O$
11	Neoisomenthol	9.430	37.89 ± 0.34	MO	$C_{10}H_{20}O$
12	2,3-Pinanediol	9.934	4.26 ± 0.34	MO	$C_{10}H_{18}O$
13	Pulegone	10.264	20.11 ± 0.40	MO	$C_{10}H_{16}O$
14	3-Carvomenthenone	10.466	0.83 ± 0.05	MO	$C_{10}H_{16}O$
15	Isobornyl acetate	10.905	0.47 ± 0.06	MO	$C_{12}H_{20}O_2$
16	Carvacrol	11.141	0.91 ± 0.07	MO	$C_{10}H_{14}O$
17	Thymol	11.297	1.41 ± 0.19	MO	$C_{10}H_{14}O$
18	Verbenone	11.822	0.46 ± 0.08	MO	$C_{10}H_{14}O$
19	Caryophyllene	12.948	1.68 ± 0.56	ST	$C_{15}H_{24}$
20	Germacrene D	13.787	0.91 ± 0.11	ST	$C_{15}H_{24}$
21	(-)-Spathulenol	15.132	0.36 ± 0.04	STO	$C_{15}H_{24}O$
22	Caryophyllene oxide	15.191	0.72 ± 0.10	STO	$C_{15}H_{24}O$
Monoterpenes (MT)			4.19%		
Oxygenated monoterpenes (MO)			92.16%		
Sesquiterpenes (ST)			2.58%		
Oxygenated sesquiterpenes (STO)			1.08%		

Antibacterial activity of CNEO and conventional antibiotics

Assessing the antimicrobial efficacy of antibacterial agents is an essential aspect of medical research. The present study analyzed the antibacterial properties of various agents, including *C. nepeta subsp. ascendens*, amoxicillin, ampicillin, and erythromycin, against different bacterial strains. The results of the minimum inhibitory concentration (MIC) tests, shown in *Table 2*, highlight the remarkable antibacterial activity of these agents. For *C. nepeta subsp. ascendens* (CNEO), minimum inhibitory concentrations range from 100 µg/ml against Escherichia coli to 1000 µg/ml against *Klebsiella pneumoniae*. For ampicillin, the MIC values ranged from 0.25 µg/ml against *Escherichia coli* and *Micrococcus luteus* to 0.75 µg/ml against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. For amoxicillin, the MIC values ranged from 0.50 µg/ml against *Micrococcus luteus* to 2 µg/ml against *Staphylococcus aureus*. Finally, for erythromycin, the MIC values ranged from 0.5 µg/ml against *Micrococcus luteus* to 1.5 µg/ml against *Staphylococcus aureus* and *Klebsiella pneumoniae.*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2205_41014114

This essential oil has a chemically diverse composition, abundant in bioactive terpene molecules. Among these constituents, Neo-iso-menthol stands out because of its significant presence. Previous studies have elucidated the bioactive properties of this molecule, in particular its antibacterial effects, as highlighted by Mirzaei et al. (2013). Furthermore, pulegone, a compound present in CNEO, has attracted particular interest due to its inhibitory properties against a broad spectrum of bacterial strains (Nozohour and Jalilzadeh-amin, 2021; Farhanghi et al., 2022). These findings corroborate the conclusions of Benkhaira et al. (2023), revealing the efficacy of essential oil from *C. nepeta* against a spectrum of pathogenic bacteria.

Table 2. *Minimum inhibitory concentration of C. nepeta subsp. ascendens essential oil (CNEO), ampicillin, amoxicillin, and erythromycin (conventional antibiotics) against different bacterial strains*

EO/Antibiotics	MIC (µg/mL)					
	S. aureus	***E. coli***	***B. subtilis***	***P. aeruginosa***	***K. pneumoniae***	***M. luteus***
CNEO	350	100	250	700	1000	700
Ampicillin	0.75	0.25	0.3	0.75	0.5	0.25
Amoxicillin	2	1	1.25	1.3	1.25	0.50
Erythromycin	1.50	1.25	0.75	0.75	1.5	0.50

(n = 3) refers to the number of repetitions for each experiment

Synergistic potential of CNEO with ampicillin

This investigation focused on analyzing the inherent antibacterial properties of combinations of *C. nepeta subsp. ascendens* and ampicillin. This evaluation included the determination of Minimum Inhibitory Concentrations (MICs) and the application of the Fractional Inhibitory Concentration Index (FICI) to assess the interaction between these agents against various bacterial strains. The results, reported in *Table 3,* concisely set out the MIC values for CNEO and ampicillin alone, as well as the FIC values for each combination studied. These data provide a quantitative understanding of the degree of interaction between the two agents, as reflected by the Fractional Inhibitory Concentration Index (FICI).

For Staphylococcus aureus, Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa, Klebsiella pneumoniae, and Micrococcus luteus, the results reveal promising synergistic activity between C. nepeta and ampicillin, highlighted by FICI values that indicate synergy or partial synergy. However, it is crucial to note that the combination did not show a significant effect against Klebsiella pneumoniae, underlining the importance of considering the specificity of interactions depending on the bacterial strain. This synergy was particularly pronounced against Staphylococcus aureus and Pseudomonas aeruginosa. The optimal synergistic effect was recorded against Pseudomonas aeruginosa with a fractional inhibitory concentration index of 0.31. Furthermore, partial synergy was observed against Bacillus subtilis (FICI 0.53), Escherichia coli (FICI 0.65), and Micrococcus luteus (FICI 0.69). Overall, this synergistic and partially synergistic interaction resulted in significant reductions, ranging from two to sixfold, in the (MIC) values of CNEO and ampicillin.

This methodical approach to assessing antimicrobial interactions offers promising prospects for the development of potentially more effective combination therapies.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2205_41014114

However, further investigations, including complementary studies, are needed to gain a better understanding of the molecular basis underlying these interactions and to optimize the clinical application of these combinations. These preliminary results provide a significant impetus for continuing in-depth research in the search for innovative solutions to the persistent threat of antimicrobial resistance.

Table 3. *Effect of combinations between C. nepeta subsp. ascendens essential oil (CNEO) and ampicillin*

Bacterial strain	Combinations	MICa (µg/mL)	MICc (µg/mL)	FIC	FICI	Interaction
S. aureus ATCC 25923	*C. nepeta* Ampicillin	350 0.75	70 0.2	0.20 0.27	0.47	Synergy
E. coli	*C. nepeta* Ampicillin	100 0.25	25 0.1	0.25 0.40	0.65	Partial synergy
B. subtilis	*C. nepeta* Ampicillin	250 0.3	50 0.3	0.20 0.33	0.53	Partial synergy
P. aeruginosa ATCC 27853	*C. nepeta* Ampicillin	700 0.75	125 0.1	0.18 0.13	0.31	Synergy
K. pneumoniae	*C. nepeta* Ampicillin	1000 0.5	600 0.3	0.60 0.60	1.20	No effect
M. luteus	*C. nepeta* Ampicillin	700 0.25	200 0.1	0.29 0.40	0.69	Partial synergy

(n = 3) refers to the number of repetitions for each experiment
MICa: MIC of single component tested alone; MICc: MIC of each component in the association at the most effective inhibition growth; FIC: Fractional inhibitory concentration is determined by the ratio MICc/MICo; FICI (fractional inhibitory concentration index): FIC of Ampicillin + FIC of CNEO

Synergistic potential of CNEO with amoxicillin

The results, recorded in *Table 4*, briefly present the Minimum Inhibitory Concentration (MIC) values for CNEO and amoxicillin in isolation, as well as the Fractional Inhibitory Concentration Index (FICI) values for each combination examined. These data provide a quantitative understanding of the nature of the interaction between these two agents, as reflected by the FICI. For *Staphylococcus aureus*, significant synergy was observed, illustrated by the FICI values indicating marked synergy (0.41). For *Escherichia coli*, partial synergy was observed, with an FICI of 0.75. For *Bacillus subtilis*, no significant effect was detected, demonstrated by an FICI of 0.96. For *Pseudomonas aeruginosa*, no significant effect was observed, with a FICI of 0.78. *Klebsiella pneumoniae* showed partial synergy, with a FICI of 0.66. Finally, *Micrococcus luteus* did not show a significant effect, with a FICI of 1.06. It is essential to emphasize that the results indicating no effect highlight the need to take into account the specificity of interactions depending on the bacterial strain. In addition, synergistic and partially synergistic interactions induced significant reductions, varying from two to four times, in the Minimum Inhibitory Concentration (MIC) values of CNEO and amoxicillin.

This methodical approach to the evaluation of antimicrobial interactions represents a rigorous and systematic approach that opens promising horizons for the development of combination therapies, which could demonstrate increased efficacy. By adopting a methodical approach, it is possible to explore in depth the various facets of interactions

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2205_41014114

between antimicrobial agents, thus gaining a better understanding of their complexity and identifying potential synergies. Therefore, this methodical approach provides a solid platform for the design of innovative and optimized combination therapies, leveraging an in-depth understanding of the mechanisms of interaction between antimicrobial agents.

Table 4. *Effect of combinations between C. nepeta subsp. ascendens essential oil and amoxicillin*

Bacterial strain	Combinations	MICa (µg/mL)	MICc (µg/mL)	FIC	FICI	Interaction
S. aureus ATCC 25923	*C. nepeta* Amoxicillin	350 2	100 0.25	0.29 0.13	0.41	Synergy
E. coli	*C. nepeta* Amoxicillin	100 1	25 0.5	0.25 0.50	0.75	Partial synergy
B. subtilis	*C. nepeta* Amoxicillin	250 1.25	200 0.2	0.80 0.16	0.96	No effect
P. aeruginosa ATCC 27853	*C. nepeta* Amoxicillin	700 1.3	275 0.5	0.39 0.38	0.78	No effect
K. pneumoniae	*C. nepeta* Amoxicillin	1000 1.25	500 0.2	0.50 0.16	0.66	Partial synergy
M. luteus	*C. nepeta* Amoxicillin	700 0.50	600 0.1	0.86 0.20	1.06	No effect

(n = 3) refers to the number of repetitions for each experiment
MICa: MIC of single component tested alone; MICc: MIC of each component in the association at the most effective inhibition growth; FIC: Fractional inhibitory concentration is determined by the ratio MICc/MICo; FICI (fractional inhibitory concentration index): FIC of Ampicillin + FIC of CNEO

Synergistic potential of CNEO with erythromycin

The findings, presented in *Table 5*, concisely depict the Minimum Inhibitory Concentration (MIC) values for CNEO and erythromycin individually, alongside the Fractional Inhibitory Concentration (FIC) values for each examined combination. These data provide a quantitative understanding of the interactions between these agents, as delineated by the FIC index.

For *Staphylococcus aureus*, pronounced synergy is discerned, elucidated by FIC values indicative of marked synergy (0.41). Regarding *Escherichia coli*, a partial synergy is evident, with an FIC of 0.74. Concerning *Bacillus subtilis*, no statistically significant effect is discerned, as demonstrated by an FIC of 1.27. For *Pseudomonas aeruginosa*, no statistically significant effect is observed, with an FIC of 0.95. *Klebsiella pneumoniae* manifests synergy, as evidenced by an FIC of 0.38. Lastly, *Micrococcus luteus* demonstrates partial synergy, with an FIC of 0.61.

It is imperative to emphasize that results indicating the absence of a statistically significant effect emphasize the need to consider the specificity of interactions based on bacterial strains. Furthermore, synergistic and partially synergistic interactions induce substantial reductions, ranging from two to four times, in the values of Minimum Inhibitory Concentration (MIC) for CNEO and erythromycin.

This systematic approach to the evaluation of antimicrobial interactions presents promising prospects for the development of potentially more effective combined therapies. comprehensive investigations, including mechanistic studies, are required to

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2205_41014114

enhance our understanding of the molecular underpinnings of these interactions and optimize their clinical application. Therefore, these preliminary findings advocate the sustained pursuit of extensive research in the quest for innovative solutions to address the persistent threat of antimicrobial resistance.

Table 5. *Effect of combinations between C. nepeta subsp. ascendens essential oil (CNEO) and erythromycin*

Bacterial strain	Combinations	MICa (µg/mL)	MICc (µg/mL)	FIC	FICI	Interaction
S. aureus ATCC 25923	*C. nepeta* Erythromycin	350 1.50	50 0.4	0.14 0.27	0.41	Synergy
E. coli	*C. nepeta* Erythromycin	100 1.25	50 0.3	0.50 0.24	0.74	Partial synergy
B. subtilis	*C. nepeta* Erythromycin	250 0.75	150 0.5	0.60 0.67	1.27	No effect
P. aeruginosa ATCC 27853	*C. nepeta* Erythromycin	700 0.75	250 0.5	0.29 0.67	0.95	No effect
K. pneumoniae	*C. nepeta* Erythromycin	1000 1.5	250 0.2	0.25 0.13	0.38	Synergy
M. luteus	*C. nepeta* Erythromycin	700 0.50	150 0.2	0.21 0.40	0.61	Partial synergy

(n = 3) refers to the number of repetitions for each experiment
MICa: MIC of single component tested alone; MICc: MIC of each component in the association at the most effective inhibition growth; FIC: Fractional inhibitory concentration is determined by the ratio MICc/MICo; FICI (fractional inhibitory concentration index): FIC of Ampicillin + FIC of CNEO

The remarkable efficacy of the antibacterial activity of the essential oil of *C. nepeta subsp. ascendens*, highlighted by these results, is of particular interest due to its distinct chemical composition, characterized by a significant abundance of terpene molecules. These compounds, such as terpenes, appear to play a crucial role in exerting the antimicrobial effect of essential oils, offering a fascinating prospect for further investigations aimed at elucidating the precise mechanisms at work. The remarkable synergistic potential of this oil does not appear to stem from chance but rather from the complex synergistic interaction between the bioactive molecules present in the oil and the active principles of the three antibiotics examined. This synergy, resulting from the coordinated collaboration of these components, confers on the whole increased efficacy against the different bacterial strains, thus underlining the relevance of this synergistic approach (Rai et al., 2017).

The combined action of the essential oil components and antibiotics appears to lead to an improvement in cell permeability. This improvement promotes the more efficient uptake of antibiotics by bacterial cells, a process that, together with the interaction of essential oil components with cell membrane channels, facilitates the optimal transport of antimicrobial agents to their intracellular targets (Solórzano-Santos and Miranda-Novales, 2012). This synergy between the secondary metabolites of essential oils and antibiotics is emerging as a promising strategy to enhance the efficacy of antibacterial treatments, opening the way to new potential clinical applications.

The promising clinical implications of these findings unequivocally justify further investigation in this area, highlighting the need for a deeper understanding of the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2205_41014114

mechanisms underlying this synergy. These findings are consistent with other research results, confirming scientific and clinical interest in the synergy between essential oils and conventional antibiotics (Jugreet and Mahomoodally, 2020; Sharma et al., 2023; Taibi et al., 2024c). This convergence also suggests a fertile avenue for further research aimed at refining this synergistic approach and exploring its practical implications in the context of combating bacterial infections.

The novel aspects of this study highlight a significant advance in research into plant-based antimicrobial agents. In particular, the results highlight *that Clinopodium nepeta subsp. ascendens* oil extract exhibits superior antibacterial efficacy to other plant-based oils, such as *Ptychotis verticillata Duby* essential oil, when applied in synergy with conventional antibiotics (Taibi et al., 2024c). This finding reinforces the relevance of *C. nepeta subsp. ascendens* oil as a potentially potent antimicrobial agent, suggesting new perspectives in the fight against bacterial infections.

Conclusion

Recent examination of essential oil extracted from *Clinopodium nepeta subs ascendens* has highlighted significant antimicrobial properties, revealing increased efficacy against specific strains of Gram-positive and Gram-negative bacteria. This discovery is generating considerable interest in medical research, as it suggests promising possibilities for the treatment of bacterial infections. The simultaneous use of CNEO in combination with conventional antibiotics such as ampicillin, amoxicillin, and erythromycin revealed synergistic interactions with certain bacterial strains. This synergy led to a significant reduction in minimum inhibitory concentrations (MICs) compared to antibiotics tested individually. These results are encouraging as they pave the way for new therapeutic strategies that could improve the efficacy of existing antibiotic treatments. This synergy not only reduces the doses needed to inhibit bacterial growth, but also helps to reduce the risk of the emergence of antibiotic-resistant strains, a major public health problem. However, despite these advances, there are still several limitations. Among these, the specificity of the interactions observed raises questions about the generalizability of the results. In addition, it is essential to broaden the range of bacterial strains studied to better understand the extent of this synergy. Furthermore, understanding the molecular mechanisms underlying this synergistic interaction is crucial for effective clinical application. In conclusion, while this study offers promising information, it also highlights the importance of further research to deepen our understanding of the benefits and limitations of using essential oils in combination with antibiotics in the treatment of microbial infections. These ongoing research efforts could shed light on new therapeutic avenues and help improve healthcare.

Acknowledgment and funding. The authors express their gratitude to the Moroccan Ministry of Higher Education, Scientific Research and Innovation, and the Hungarian Ministry of Innovation and Technology for supporting this work under the Morocco-Hungary project cooperation. The authors would like to extend their sincere appreciation to the Researchers Supporting Project , King Saud University, Riyadh, Saudi Arabia for funding this work through the project number (RSP-2024R437).

Conflict of interests. The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Data availability. Data will be available upon request from the corresponding author.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2205_41014114

REFERENCES

[1] Al-Mijalli, S. H., Mrabti, N. N., Ouassou, H., Sheikh, R. A., Abdallah, E. M., Assaggaf, H., Bakrim, S., Alshahrani, M. M., Awadh, A. A., Al, Qasem, A., Attar, A., Lee, L. H., Bouyahya, A., Goh, K. W., Ming, L. C., Mrabti, H. N. (2022): Phytochemical variability, in vitro and in vivo biological investigations, and in silico antibacterial mechanisms of Mentha piperita essential oils collected from two different regions in Morocco. – Foods 11(21): 1-22. https://doi.org/10.3390/foods11213466.

[2] Beddiar, H., Boudiba, S., Benahmed, M., Tamfu, A. N., Ceylan, Ö., Hanini, K., Kucukaydin, S., Elomri, A., Bensouici, C., Laouer, H. (2021): Chemical composition, anti-quorum sensing, enzyme inhibitory, and antioxidant properties of phenolic extracts of Clinopodium nepeta L. Kuntze. – Plants 10(9): 1955.

[3] Bengtsson-Palme, J., Kristiansson, E., Larsson, D. G. J. (2018): Environmental factors influencing the development and spread of antibiotic resistance. – FEMS Microbiology Reviews 42(1): fux053.

[4] Benkhaira, N., El Hachlafi, N., Jeddi, M., Abdnim, R., Bnouham, M., Koraichi, S. I., Fikri-Benbrahim, K. (2023): Unveiling the phytochemical profile, in vitro bioactivities evaluation, in silico molecular docking and ADMET study of essential oil from Clinopodium nepeta grown in Middle Atlas of Morocco. – Biocatalysis and Agricultural Biotechnology 102923.

[5] Cherrat, L., Espina, L., Bakkali, M., Pagán, R., Laglaoui, A. (2014): Chemical composition, antioxidant and antimicrobial properties of Mentha pulegium, Lavandula stoechas and Satureja calamintha Scheele essential oils and an evaluation of their bactericidal effect in combined processes. – Innovative Food Science and Emerging Technologies 22: 221-229. https://doi.org/10.1016/j.ifset.2013.12.016.

[6] Chokshi, A., Sifri, Z., Cennimo, D., Horng, H. (2019): Global contributors to antibiotic resistance. – Journal of Global Infectious Diseases 11(1): 36.

[7] Clark, L. (2000): Antibiotic resistance: a growing and multifaceted problem. – British Journal of Nursing 9(4): 225-230.

[8] Debbabi, H., El Mokni, R., Chaieb, I., Nardoni, S., Maggi, F., Caprioli, G., Hammami, S. (2020): Chemical composition, antifungal and insecticidal activities of the essential oils from Tunisian Clinopodium nepeta subsp. nepeta and Clinopodium nepeta subsp. glandulosum. – Molecules 25(9): 2137.

[9] Dhingra, S., Rahman, N. A. A., Peile, E., Rahman, M., Sartelli, M., Hassali, M. A., Islam, T., Islam, S., Haque, M. (2020): Microbial resistance movements: an overview of global public health threats posed by antimicrobial resistance, and how best to counter. – Frontiers in Public Health 8: 535668.

[10] Didry, N., Dubreuil, L., Pinkas, M. (1993): Microbiological properties of protoanemonin isolated from Ranunculus bulbosus. – Phytotherapy Research 7(1): 21-24.

[11] El Atki, Y., Aouam, I., El Kamari, F., Taroq, A., Nayme, K., Timinouni, M., Lyoussi, B., Abdellaoui, A. (2019): Antibacterial activity of cinnamon essential oils and their synergistic potential with antibiotics. – Journal of Advanced Pharmaceutical Technology & Research 10(2): 63.

[12] El Bouzidi, A., Taibi, M., Laarej, S., El Hassania, L., Haddou, M., Hachlafi, N. El, Naceiri Mrabti, H., Baraich, A., Bellaouchi, R., Asehraou, A. (2024): Chemical profiling of volatile compounds of the essential oil of grey-leaved rockrose (Cistus albidus L.) and its antioxidant, anti-inflammatory, antibacterial, antifungal, and anticancer activity in vitro and in silico. – Frontiers in Chemistry 12: 1334028.

[13] El Guerrouj, B., Taibi, M., Elbouzidi, A., Bouhassoun, S., Loukili, E. H., Moubchir, T., Haddou, M., Hammouti, Y., Khoulati, A., Addi, M. (2023): The Effect of altitude on the chemical composition, antioxidant and antimicrobial activities of Eucalyptus globulus Labill. essential oils. – Tropical Journal of Natural Product Research 7(11).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2205_41014114

[14] Elbouzidi, A., Ouassou, H., Aherkou, M., Kharchoufa, L., Meskali, N., Baraich, A., Mechchate, H., Bouhrim, M., Idir, A., Hano, C., Zrouri, H., Addi, M. (2022): No Title. – Pharmaceuticals 15(9): 1156. https://doi.org/10.3390/ph15091156.
[15] Etebu, E., Arikekpar, I. (2016): Antibiotics: classification and mechanisms of action with emphasis on molecular perspectives. – Int. J. Appl. Microbiol. Biotechnol. Res 4(2016): 90-101.
[16] Farhanghi, A., Aliakbarlu, J., Tajik, H., Mortazavi, N., Manafi, L., Jalilzadeh-Amin, G. (2022): Antibacterial interactions of pulegone and 1 8-cineole with monolaurin ornisin against Staphylococcus aureus. – Food Science & Nutrition 10(8): 2659-2666.
[17] French, G. L. (2005): Clinical impact and relevance of antibiotic resistance. – Advanced Drug Delivery Reviews 57(10): 1514-1527.
[18] Gould, K. (2016): Antibiotics: from prehistory to the present day. – Journal of Antimicrobial Chemotherapy 71(3): 572-575.
[19] Haddou, M., Taibi, M., Elbouzidi, A., Loukili, E. H., Yahyaoui, M. I., Ou-Yahia, D., Mehane, L., Addi, M., Asehraou, A., Chaabane, K., Bellaouchi, R., El Guerrouj, B. (2023): Investigating the impact of irrigation water quality on secondary metabolites and chemical profile of Mentha piperita essential oil: analytical profiling, characterization, and potential pharmacological applications. – International Journal of Plant Biology 14(3): 638-657. https://doi.org/https://doi.org/10.3390/ijpb14030049.
[20] Jugreet, B. S., Mahomoodally, M. F. (2020): Essential oils from 9 exotic and endemic medicinal plants from Mauritius shows in vitro antibacterial and antibiotic potentiating activities. – South African Journal of Botany 132: 355-362.
[21] Langeveld, W. T., Veldhuizen, E. J. A., Burt, S. A. (2014): Synergy between essential oil components and antibiotics: a review. – Critical Reviews in Microbiology 40(1): 76-94.
[22] Leghari, F., Kamboh, A. A., Leghari, R. A., Korejo, N. A., Malhi, K. K. (2021): Antimicrobial potential of tea tree, clove, basil and thyme essential oils against bacterial isolates of bovine wounds. – JAPS: Journal of Animal & Plant Sciences 31(5).
[23] Loukili, E. H., Abrigach, F., Bouhrim, M., Bnouham, M., Fauconnier, M. L., Ramdani, M. (2021): Chemical composition and physicochemical analysis of Opuntia dillenii Extracts grown in Morocco. – Journal of Chemistry 2021. https://doi.org/10.1155/2021/8858929.
[24] Loukili, E. H., Ouahabi, S., Elbouzidi, A., Taibi, M., Yahyaoui, M. I., Asehraou, A., Azougay, A., Saleh, A., Al Kamaly, O., Parvez, M. K., El Guerrouj, B., Touzani, R., Ramdani, M. (2023): Phytochemical composition and pharmacological activities of three essential oils collected from eastern Morocco (Origanum compactum, Salvia officinalis, and Syzygium aromaticum): a comparative study. – Plants 12(19): 3376. https://doi.org/10.3390/plants12193376.
[25] Maxson, T., Mitchell, D. A. (2016): Targeted treatment for bacterial infections: prospects for pathogen-specific antibiotics coupled with rapid diagnostics. – Tetrahedron 72(25): 3609-3624.
[26] Mirzaei, H. H., Faraji, R., Hooshidari, F., Bigdelo, M., Rezaei, K. (2013): Evaluation of phytochemical composition of Opsicarpium insignis Mozaff. from Iran by nano scale injection techniques. – Evaluation 5(4): 1911-1914.
[27] Nozohour, Y., Jalilzadeh-amin, G. (2021): Comparison of antibacterial activity of trans-cinnamaldehyde 1 8 cineole, and pulegone against Streptococcus equi subsp equi isolated from horse. – Iranian Journal of Medical Microbiology 15(5): 606-611.
[28] Öztürk, G., Yilmaz, G., Gülnur, E., Demirci, B. (2021): Chemical composition and antibacterial activity of Clinopodium nepeta subsp. glandulosum (Req.) Govaerts essential oil. – Natural Volatiles and Essential Oils 8(3): 75-80.
[29] Rai, M., Paralikar, P., Jogee, P., Agarkar, G., Ingle, A. P., Derita, M., Zacchino, S. (2017): Synergistic antimicrobial potential of essential oils in combination with nanoparticles: emerging trends and future perspectives. – International Journal of Pharmaceutics 519(1-2): 67-78.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2205_41014114

[30] Raveau, R., Fontaine, J., Lounès-Hadj Sahraoui, A. (2020): Essential oils as potential alternative biocontrol products against plant pathogens and weeds: a review. – Foods 9(3): 365.
[31] Rodenak-Kladniew, B., Castro, M. A., Gambaro, R. C., Girotti, J., Cisneros, J. S., Viña, S., Padula, G., Crespo, R., Castro, G. R., Gehring, S. (2023): Cytotoxic screening and enhanced anticancer activity of Lippia alba and Clinopodium nepeta essential oils-loaded biocompatible lipid nanoparticles against lung and colon cancer cells. – Pharmaceutics 15(8): 2045.
[32] Rosato, A., Sblano, S., Salvagno, L., Carocci, A., Clodoveo, M. L., Corbo, F., Fracchiolla, G. (2020): Anti-biofilm inhibitory synergistic effects of combinations of essential oils and antibiotics. – Antibiotics 9(10): 637.
[33] Sharma, N., Sheikh, Z. N., Alamri, S., Singh, B., Kesawat, M. S., Guleria, S. (2023): Chemical composition, antibacterial and combinatorial effects of the essential oils from Cymbopogon spp., Mentha arvensis with conventional antibiotics. – Agronomy 13(4): 1091.
[34] Solórzano-Santos, F., Miranda-Novales, M. G. (2012): Essential oils from aromatic herbs as antimicrobial agents. – Current Opinion in Biotechnology 23(2): 136-141.
[35] Taibi, M., Elbouzidi, A., Ouahhoud, S., Loukili, E. H., Ou-Yahya, D., Ouahabi, S., Alqahtani, A. S., Noman, O. M., Addi, M., Bellaouchi, R., Asehraou, A., Saalaoui, E., El Guerrouj, B., Chaabane, K. (2023a): Evaluation of antioxidant activity, cytotoxicity, and genotoxicity of Ptychotis verticillata essential oil: towards novel breast cancer therapeutics. – Life 13(7). https://doi.org/10.3390/life13071586.
[36] Taibi, M., Elbouzidi, A., Ou-Yahia, D., Dalli, M., Bellaouchi, R., Tikent, A., Roubi, M., Gseyra, N., Asehraou, A., Hano, C., Addi, M., El Guerrouj, B., Chaabane, K. (2023b): Assessment of the antioxidant and antimicrobial potential of Ptychotis verticillata Duby essential oil from eastern Morocco: an in vitro and in silico analysis. – Antibiotics 12(4). https://doi.org/10.3390/antibiotics12040655.
[37] Taibi, M., Loukili, E. H., Elbouzidi, A., Baraich, A., Haddou, M., Bellaouchi, R., Saalaoui, E., Asehraou, A., Addi, M., Bourhia, M. (2024a): Exploring the pharmacological potential of the chemically characterized essential oil from Clinopodium nepeta subsp. ascendens: a combined in vitro and in silico analysis. – Moroccan Journal of Chemistry 12(3): 997-1021.
[38] Taibi, M., Elbouzidi, A., Haddou, M., Baraich, A., Loukili, E. H., Moubchir, T., Allali, A., Bellaouchi, R., Asehraou, A., Addi, M. (2024b): Phytochemical characterization and multifaceted bioactivity assessment of essential oil from Ptychotis verticillata Duby: anti-diabetic, anti-tyrosinase, and anti-inflammatory activity. – Heliyon 10(8). https://doi.org/10.1016/j.heliyon.2024.e29459.
[39] Taibi, M., Elbouzidi, A., Haddou, M., Loukili, E. H., Bellaouchi, R., Asehraou, A., Douzi, Y., Addi, M., Salamatullah, A. M., Nafidi, H.-A. (2024c): Chemical profiling, antibacterial efficacy, and synergistic actions of Ptychotis verticillata Duby essential oil in combination with conventional antibiotics. – Natural Product Communications 19(1): 1934578X231222785.
[40] Vlachou, G., Papafotiou, M., Daferera, D. J., Tarantilis, P. A. (2023): Yield and composition of the essential oil of Clinopodium nepeta subsp. Spruneri as affected by harvest season and cultivation method, ie, outdoor, greenhouse and in vitro culture. – Plants 12(24): 4098.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2205_41014114