

## Original Research Article

# Chemical composition and biological activities of n-butanol extract of *Lepidium sativum* L (Brassicaceae) seed

Ouahiba Ait-yahia<sup>1</sup>, François Perreau<sup>2</sup>, Samia-Aichouche Bouzroua<sup>3</sup>, Yamina Benmalek<sup>4</sup>, Tahar Dob<sup>5</sup>, Aicha Belkebir<sup>1</sup>

<sup>1</sup>Laboratory of Vegetale Physiology, Faculty of Biological Science, Houari Boumediene University of Sciences and Technology, BP 31, El-Alia, Bab-Ezzouar 16111, Algiers, Algeria, <sup>2</sup>Institut Jean-Pierre Bourgin, INRA, Agro Paris Tech, CNRS, Université Paris-Saclay, 78000 Versailles, France, <sup>3</sup>Laboratory of Applied Organic Chemistry, <sup>4</sup>Laboratory of Microbiology, Faculty of Biological Science, Houari Boumediene University of Sciences and Technology, BP 31, El-Alia, Bab-Ezzouar 16111, <sup>5</sup>Laboratory of Research for Bioactive Products, École Normale Supérieure (ENS-Kouba), Algiers, Algeria

\*For correspondence: **Email:** [hiba.ait357@yahoo.com](mailto:hiba.ait357@yahoo.com); **Tel:** +213 552732833

Sent for review: 29 September 2017

Revised accepted: 30 April 2018

## Abstract

**Purpose:** To investigate the chemical composition, antioxidant properties and antibacterial effects of n-butanol extract of *Lepidium sativum* (Brassicaceae) seed.

**Methods:** The antioxidant activity of extracts of the seed obtained by maceration was determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH), reducing power, and  $\beta$ -caroten/linoleic acid assays. The antimicrobial activity of the extract was tested against five strains of pathogenic microorganisms, viz, *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 43300, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25992 and *Pseudomonas aeruginosa* ATCC 27852. Its phytochemical composition was determined by liquid chromatography-mass spectrometry (LC-MS).

**Results:** A total of 17 compounds were identified by LC-MS, among which four compounds were kaempferol-hexose-rhamnose and three derivatives of kaempferol-rhamnose-hexose (benzoyl hexose) were previously unknown. The extract had better antioxidant than antibacterial properties.

**Conclusion:** These results indicate that n-butanol extract of *Lepidium sativum* seeds possesses good antioxidant properties which are most likely due to the presence of quercetin and kaempferol. Thus, these compounds account for the use of *Lepidium sativum* in traditional medicine.

**Keywords:** *Lepidium sativum*, Quercetin, Kaempferol, Antioxidant, Antibacterial

This is an Open Access article that uses a funding model which does not charge readers or their institutions for access and distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0>) and the Budapest Open Access Initiative (<http://www.budapestopenaccessinitiative.org/read>), which permit unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited.

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

## INTRODUCTION

Medicinal plants have continued to receive a lot of attention from researchers due to their pharmacological effects such as antioxidant, anti-inflammatory and antibacterial properties. The resistance to antibiotics by pathogenic microorganisms in a public health problem in the

world. Indeed, many bacterial strains show resistance to several groups of antibiotics [1]. However, in the beginning of the 20th century, scientists have begun to search other molecules with antibacterial activity. Medicinal plants are natural and safer sources of anti-free radical compounds. Reactive oxygen species (ROS) provoke oxidative damage which plays a role in

the pathogenesis of diseases such as cancer, atherosclerosis and Alzheimer's [2,3].

Brassicaceae is a large family made up of vegetables (Brassica crops), medicinal plants (*Capsella bursa-pastoris*), oil-rich plants (*Brassica napus*) and the model spices of plant science (*Arabidopsis thaliana*). Among these plants, the seeds of *Lepidium sativum* (garden cress) are widely used in Arabic countries for their medicinal properties. They have various nutritional and medicinal attributes, and are recommended for anti-diarrheal [4], cardiogenic, hypotensive, antimicrobial, bronchodilator and hypoglycemic applications [5]. These activities can be attributed to bioactive compounds as flavonoids present in this plant. Flavonoids (C6-C3-C6) can be classified into different sub-classes (flavones, flavanones, flavonols, isoflavones, flavanols, chalcones and anthocyanins) and they are commonly found conjugated to sugars in the form of O-glycosides or C-glycosides forms [6].

Reactive oxygen species (ROS) provoke oxidative damage which plays a role in the pathogenesis of diseases such as cancer, atherosclerosis and Alzheimer's disease [2, 3].

Brassicaceae is a large family made up of vegetables (Brassica crops), medicinal plants (*Capsella bursa-pastoris*), oil-rich plants (*Brassica napus*) and the model spices of plant science (*Arabidopsis thaliana*). Among these plants, seeds of *Lepidium sativum* (garden cress) are widely used in Arabic countries for their medicinal properties. They have various nutritional and medicinal attributes, and are recommended for anti-diarrheal [4], cardiogenic, hypotensive, antimicrobial, bronchodilator and hypoglycemic applications [5]. These activities can be attributed to bioactive compounds, such as flavonoids, present in this plant. Flavonoids (C6-C3-C6) can be classified in different subclasses (flavones, flavanones, flavonols, isoflavones, flavanols, chalcones and anthocyanins) and they are commonly found conjugated to sugars in the form of O-glycosides or C-glycosides [6]. Several studies have been done on *Lepidium sativum* seeds. Recently, two new acylated kaempferol and quercetin compounds isolated from the seeds of *Lepidium sativum* [7]. In this study, the phytochemical composition, antioxidant properties and antibacterial activities of n-butanol extract of *Lepidium sativum* seeds were investigated.

## EXPERIMENTAL

### Plant seed collection

Seeds of *L. Sativum* were collected from an area in the Northwest of Algeria in May 2012. The

seeds were identified by Dr H. Hadj-Arab of the laboratory of Eco-genetic and Biosystemics, Houari Boumediene, according to the flora of Algeria. The identification was done in the herbarium of Botany Department, Ecole Nationale Supérieure Agronomique d'Alger (ENSA), and a voucher specimen no. P12 was assigned.

### Extract preparation

Twenty grams of the seeds were extracted three times with 70 % methanol for 48 h. The extract was then filtered through Whatman No.2 paper. Two more 20g batches were similarly extracted, and the combined extract was concentrated in vacuum at 40 °C. It was extracted with different solvents (50 mL each) in order: hexane, ethyl-ether, ethyl-acetate and n-butanol. Each solvent extract was evaporated and taken up in methanol. Only the n-butanol fraction was used for the identification of phytochemical composition, as well as for antimicrobial and antioxidant assays.

### LC-MS analysis of flavonoids

The extract was injected into the ESI source using a Waters Acquity I-Class separation module with an Acquity PDA UV detector. Separation was achieved on an Uptisphere C18 ODB, 100 x 2.1 mm. The flow rate was 0.4 ml/min, with elution gradient of 0.1 % acetic acid (A) and 0.5 % acetic acid (B) in acetonitrile, in the order 0 – 2 min 5 % A, 4min 10 % A, 17min 40 % A, 21–23min 100 % B, 23 – 28min 5 % A. The components were identified and quantified at positive and negative ESI modes using triple quadrupole mass spectrometer (Xevo TQ-S, Waters) at full scan. The operating conditions used were: capillary and extraction voltages of 2.80kV and 3V, respectively; with gas desolvation and source block temperatures fixed at 150 and 300 °C, respectively. Desolvation and nebulisation were facilitated using nitrogen gas at flow rates of 200 and 1000L/h, respectively.

### Determination of total phenolic contents

The levels of total phenolic compounds in the extract were quantified colorimetrically as gallic acid equivalents (GAE) with Folin-Ciocalteu reagent as described earlier [8]. The results were expressed as GAE/gram dry weight.

### Determination of minimum inhibitory concentration (MIC) of extract

Antimicrobial test was carried out on five reference strains: *S. aureus* (ATCC 25923, methicillin-resistant), *S. aureus* (ATCC 43300 methicillin-sensitive), *E. faecalis* (ATCC 29212),

*E. coli* (ATCC 25992), and *P. aeruginosa* (ATCC27852). The MIC and IC<sub>50</sub> values for the extract were determined using the serial broth dilutions method in sterile tubes [1,9,10]. The ranges of final extract concentrations used were 0.05- 32mg/mL. Each tube was inoculated with a standardized inoculum of 10<sup>6</sup> cells. This inoculum was obtained by diluting 2-3 well-isolated colonies on nutrient agar in 10 mL of nutrient broth. Following homogenization, the diluted colonies were placed in an incubator for 3 h at 37 °C. Thereafter, one milliliter of each culture medium was introduced into 10mL of nutrient broth in a tube containing the extract. The tubes were assessed for turbidity as evidence of bacterial growth After incubation at 37 °C for 24 h, And MIC was calculated in terms of the lowest extract concentration that produce 100% growth suppression. Unexposed organisms were used as control.

#### Determination of *in vitro* antioxidant activity

##### DPPH method

The method of Brand-Williams *et al* [11] was used. To 1mL of extract was added 2mL of 0.4 mM DPPH in methanol. The mixture was incubated in the dark for 30min, and the absorbance was read at 517nm. All determinations were performed in triplicate. The DPPH scavenging activity (D) was calculated using Eq 1.

$$D (\%) = \{(Ac As)/Ac\}100 \dots\dots\dots (1)$$

##### Reducing power (FRAP) method

Reducing power (FRAP) was evaluated using the potassium ferricyanide colorimetric method as described by Oyaizu [12].

##### $\beta$ -Carotene/linoleic acid method

$\beta$ -Carotene/linoleic acid spectrophotometric method was used to estimate the antioxidant activities of the extract [13]. The principle of this procedure is that the substrate  $\beta$ -carotene undergoes color change as a result of linoleic acid oxidation. The decrease in absorbance is measured after 120min at 470nm. In the assay, an aliquot of the extract solution (0.2mL) was mixed thoroughly with 4.8mL of linoleic acid emulsion and the absorbance was read at 470nm, with butylated hydroxyl toluene (BHT) as standard. The % inhibition of  $\beta$ -carotene bleaching (B) was calculated as relative antioxidant activity (RAA) [14] using Eq 2.

$$B (\%) = \{(A_i/A_{BHT})\}100 \dots\dots\dots (2)$$

where  $A_i$  = absorbance of the test sample, and  $A_{BHT}$  = absorbance of the standard control (BHT)

#### Statistical analysis

The data are presented as mean  $\pm$  SD and were analyzed by one-way analysis of variance (ANOVA) using Statistica software, followed by Tukey's multiple range tests.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Compounds identified in *Lepidium sativum* seed extract

Seventeen Compounds were identified from the seed extract of *Lepidium sativum* by LC-MS, as shown in Table 1.

These compounds included glucotropaeoline (glucosinolate), sinapoyl malate, sinapic acid, sinapine, sinapoyl diglucose and ferulic acid., Glucotropaeoline was the most abundant component (67.5 %) followed by sinapine (13.43 %). In addition, *Lepidium sativum* contained relatively high concentrations of flavonoids. From the results obtained, quercetin and kaempferol were the main flavonols present, with varying degrees of glycosylation (mono, di and tri-glycoside). Many isomers of hexose rhamnoside and other derivatives were also present. All the glycosides were attached to a rhamnose.

Four components (heterosides) were identified for the first time. According to their HPLC retention times and MS profiles, these heterosides were identified as two isomers of kaempferol hexose rhamnose (ion at  $m/z$  595 [M-H]<sup>+</sup>) and three derivatives of kaempferol rhamnose\_hexose (benzoyl-hexose) (ion at 861  $m/z$  [M-H]<sup>+</sup>). The other compounds have been previously identified [15].

Kaempferol di-hexoside rhamnose and quercetin di-hexoside rhamnose were the most important heterosides present in *L. sativum* (at high levels 3.77 and 2.07 %, respectively).

### Antioxidant activity and total phenolic contents of the extract

The results are presented in Table 2. The IC<sub>50</sub> of the n-butanol extract in the DPPH radical scavenging assay was 67.1 $\pm$ 0.3  $\mu$ g/mL. Reducing power (FRAP) and % inhibition of  $\beta$ -carotene bleaching were 100 $\pm$ 0.05  $\mu$ g.mL<sup>-1</sup> and 54.91%, respectively. The antioxidant activity of the butanolic extract was < the standards (ascorbic acid, quercetin and  $\alpha$ -tocopherol).

**Table 1:** Quantitative and qualitative characterization of phytochemical compounds from *L. sativum*

Compound	RT	Molecular ion MH-	Molecular ion MH+	Proportion crude extract	Compound (%)
Glucopaoline	-3.43	408, 410	/	187.12	67.507
Sinapoyl malate	-7.73	339, 223	207	4.62	1.67
Ferulic acid	-7.95	193	195	0.38	0.137
Sinapic acid	-8.15	223	225	1.53	0.552
Sinapoyl di-glucose	-12.9	573,223	575	7.41	2.675
Sinapine	+5.97	/	310	37.2	13.43
Q di-hexose rhamnose	+5.99	771	773	5.73	2.07
K di-hexose rhamnose	+6.4	755	757	10.46	3.77
K hexose rhamnose 1	+7.15	/	595	4.68	1.69
Q hexose rhamnose	+7.35	609	611	0.17	0.06
K hexose rhamnose 2	+7.75	593	595	0.26	0.837
K rhamnose (benzo) di-hexose 1	+8.84	859	861	2.96	1.867
K rhamnose (benzo) di-hexose 2	+9.25	859	861	0.31	0.11
K hexose rhamnose 3	+10.19	/	595	1.57	0.566
Q rhamnose	+10.94	447	449	0.51	0.322
K rhamnose (benzo) di-hexose 3	+11.01	859	861	0.98	0.35
K rhamnose	+12.45	431	433	0.29	0.1

Q = quercetin, K = kaempferol

**Table 2:** Total phenolic content expressed as mg/g gallic acid equivalent (EAG), and antioxidant activity of n-butanol extract *L. sativum* seeds

Material	Total polyphenols mg/gEAG	Antioxidant activity		
		DPPH (IC <sub>50</sub> ( $\mu\text{g}\cdot\text{mL}^{-1}$ ))	RP ( $\mu\text{g}\cdot\text{mL}^{-1}$ )	$\beta$ -Caroten(%)
Extract	1.629 $\pm$ 0.13	67.1 $\pm$ 0.3*	100 $\pm$ 0.051	54.91 $\pm$ 0.116*
Ascorbic acid	-	4.04 $\pm$ 0.13*	7.53 $\pm$ 0.104*	30.55 $\pm$ 0.117*
Quercetin	-	6.72 $\pm$ 0.158*	20 $\pm$ 0.01*	45.37 $\pm$ 0.12**
$\alpha$ -Tocopherol	-	-	-	98.55 $\pm$ 0.248*

Values (mean  $\pm$  SD, n = 3); \*significant difference ( $p < 0.05$ )

**Table 3:** Antibacterial activity of n-butanol extract *L. sativum* seeds

Parameter	Antibacterial activity (mg.mL <sup>-1</sup> )				
	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus 1</i>	<i>S. aureus 2</i>	<i>E. faecalis</i>
IC <sub>50</sub>	0.41	0,42	0,47	0,42	0,4
MIC	5	4	4	4	3,5

### Antibacterial activity

The results of antibacterial activity of *Lepidium sativum* which was evaluated *in vitro* against five bacterial strains Table 2. The calculated MIC and IC<sub>50</sub> are presented in the Table 3. The n-butanol fraction has exhibited appreciable antibacterial activity against all the bacteria strains tested irrespective of Gram strain.

### DISCUSSION

The main flavonols present in many Brassicaceae species are quercetin, kaempferol and their derivatives (mono-, di-, tri-glycosides) [16,17]. This is in agreement with the results obtained in the present study. A previously unknown quercetin-3-O-(6-O-benzoyl)- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 3)- $\beta$ -D-galactopyranoside-7-O- $\alpha$ -L rhamnopyranoside was identified by Fan et al [7]. However, while the same acylation was

present in the Algerian variety, quercetin was replaced by a kaempferol. The results available in the literature regarding the phytochemical compositions of leaves of *Lepidium sativum* and leaves of other Brassicaceae plants indicate the presence of three flavonols viz quercetin, kaempferol and isorhamnetin [18]. Thus, it seems that isorhamnetin is absent in the seeds.

Results from various studies indicate that all flavonoids glycosides possess strong antimicrobial activity against strains of *P. aeruginosa* and *S. aureus* but exhibit a very low activity against *E. coli* [19]. This is also in agreement with the results of antimicrobial activity of the n-butanol extract used in this study. Studies on methanol and chloroform extract of *Lepidium sativum* have also shown low antibacterial activity against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* [20]. The antibacterial activity of

flavonol glycosides towards Gram-positive bacteria may be related to the cell membrane constituents and structures of these organisms. The flavonols may also be inhibitory to the activities of cellular enzymes. It has been also reported that flavonol glycosides chelate metals, an effect which can inhibit some enzymes [21]. It is important to mention that the permeability of the bacterial cells to the tested compounds is one of the factors that determine their antibacterial effects.

The flavonols have the ability to donate hydrogen ions to synthetic free radical compound (DPPH) [22], and to reduce potassium ferricyanide ( $\text{Fe}^{3+}$ ) to potassium ferrocyanide ( $\text{Fe}^{2+}$ ) by donating an electron [23]. These antioxidants can stop the conversion of linoleic hydro-peroxide or eliminate this peroxide [24]. The differential activities of flavonoids could depend on the solvent used for extraction and on the position of substituents in the flavonoid nucleus. These differences in substituents give rise to the major bioactive compounds such as quercetine, kampferol and their glycosides which are present in *Lepidium sativum* seeds [7,15-17].

## CONCLUSION

The results of this study indicate that n-butanol extract of *L. sativum* seed possesses significant antioxidant properties, and significant antibacterial activities against selected Gram-positive and Gram-negative pathogens. Further investigations are needed to elucidate the structures of the identified compounds in detail using nuclear magnetic resonance (NMR). It would also be helpful to carry out *in vivo* tests on these compounds in their isolated and purified forms.

## DECLARATIONS

### Conflict of Interest

No conflict of interest associated with this work.

### Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

## REFERENCES

1. Traoré Y, Ouattara K, Yéo D, Doumbial, Coulibaly A. Recherche des activités antifongique et antibactérienne des feuilles d'*Annona senegalensis* Pers. (Annonaceae). *J Applied Biosci* 2012; 58: 4234–4242.
2. Shen SS, Callaghan D, Juzwik C, Xiong HQ, Huang PL, Zhang WD. ABCG2 reduces ROS-mediated toxicity and inflammation: A potential role in Alzheimer's disease. *J Neurochem* 2010; 114:1590-1604.
3. Mullen W, Marks SC, Crozier A. Evaluation of phenolic compounds in commercial fruit juices and fruit drinks. *J Agric Food Chem* 2007; 55: 3148-3157.
4. Merzouki A, Ed-derfoufi F, Molero MJ. Contribution to the knowledge of Rifian traditional medicine. II: Folk medicine in Ksar Lakbir District (NW Morocco). *Fitoterapia* 2000; 71: 278-307.
5. Edwards S, Asfaw Z. Plants used in Africa traditional medicine as practice in Ethiopia and Uganda. NAPRECA: Addis Ababa University, Addis Ababa; 1992.
6. Xiao J, Muzashviti TS, Georgiev MI. Advances in the biotechnological glycosylation of valuable flavonoids. *Biotechnol Adv* 2014; 32: 1145-1156.
7. Fan QL, Zhu YD, Huang WH, Qi Y, Guo BL. Two new acylated flavonols glycosides from seeds of *Lepidium sativum*. *Molecules* 2014; 19: 11341-11349.
8. Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am J Enol Vitic* 1965; 16: 144-153.
9. Aldebasi et al. 2013 AldebasiYH, Aly SM, Qureshi MA, Khadri H. Novel antibacterial activity of *Terfizia claveryi* aqueous extract against clinical isolates of corneal ulcer. *Afr J Biotechnol* 2013; 12: 6340-6346.
10. Rubens DM, Constantin OO, Moevi AA, Guessennnd KN, Daouda T, Nguessan JD, Dosso M, Djaman AJ. Anti-*Staphylococcus aureus* activity of the aqueous extract and hexanic fraction of *Thonningia sanguine*. *Int J Pharmacogn Phytochem Res* 2015; 7: 301-306.
11. Brand-Williams W, Cuvelier ME, Berest FO. Use of free radical method to evaluate antioxidant activity. *Leben Wiss Technol* 2008; 28: 25–30.
12. Oyaizu M. Studies on products of the browning reaction antioxidative activities of browning reaction products prepared from glucosamine. *Jap J Nutr* 1986; 44: 307-315.
13. Jayaprakasha GK, Singh RP, Sakariah KK. Antioxidant activity of grape seed (*Vitis vinifera*) extracts on peroxidation models *in vitro*. *Food Chem* 2001; 73: 285-290.
14. Tepe B, Sokmen M, Akpulute HA, Sokmen A. Screening of Antioxidant potential of six *Salvia* species. *Food Chem* 2006; 95: 200-204.
15. Oszmianski J, Kolniak OJ, Wojdylo A. Application of ultraperformance liquid chromatography-photodiode detector-quadrupole/ time of light-mass spectrometry (UPLC-PDA-Q/TOF-MS) method for the characterization of phenolic compounds of *Lepidium sativum* L. sprouts. *European Food Research and Technology* 2013; 236: 699-706.
16. Cartea ME, Fransisco M, Soengas P, Velasco P. Phenolic compounds in *Brassica* vegetables. *Molecules* 2011; 16: 251-280.

17. Zia-Ul-Haq M, Ahmad S, Calani L, Mazzeo T, Del Rio D, Pellegrini N, De Feo V. Compositional study and antioxidant potential of *Ipomoea hederacea* Jacq. And *Lepidium sativum* L. seeds. *Molecules* 2012 ; 17 : 10306-10321
18. Bell L, Oruna-Concha MJ, Wagstaff C. Identification and quantification of glucosinolate and flavonol compounds in rocket salad (*Eruca sativa*, *Eruca vesicaria* and *Diplotaxis tenuifolia*) by LC–MS: High lighting the potential for improving nutritional value of rocket crops. *Food Chem* 2015; 172: 852–861.
19. Xiao JB, Chen TT, Cao H. Flavonoide glycosylation and biological benefits. *Biotechnol Adv* 2014; 32:1145-1156.
20. Abuelgasim AI, Ali MI, Hassan A. Antimicrobial activities of extracts for some of medicinal plants. *International Journal of Advanced and Applied Sciences* 2015; 2: 1-5.
21. Ouattara K, Doumbia I, Touré A, Djaman AJ, Coulibaly A. Activité antibactérienne des extraits des feuilles de *Morinda morindoides* (*Morinda*, *Rubiaceae*) sur *Staphylococcus aureus* et *Pseudomonas aeruginosa*. *Pharmacognosie infectiologie* 2013; 11: 172-177.
22. Pereira DM, Valentao P, Pereira JA, Andrade PB. Phenolics: From Chemistry to Biology. *Molecules* 2009; 14: 2202-2211.
23. Shah NA, Khan MR, Ahmad B, Noureen F, Rashid U, Khan RA. Investigation on flavonoid composition and anti-free radical potential of *Sidacordata*. *BMC Complem Altern Med* 2013; 13: 276.
24. Golivand MB, Nasrabadi MR, Batooli H, Ebrahimabadi AH. Chemical composition and antioxidant activities of the essential oil and methanolic extracts of *Psammogeton canescens*. *Food Chem Toxicol* 2010; 48: 24-28.