Variability of phenolic compounds accumulation and bioactivity among Tunisian *Artemisia arborescens* L. genetic resources

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Abstract

The antibiotic resistance emergence poses a serious threat for plants, humans and animals health and resulted in significant economic costs. Plant natural products present actually an alternative for the development of antimicrobial agents. In this study the phytochemical characterisation of the hydro-ethanolic extracts of four Artemisia arboresccens L. populations in Tunisia and their antimicrobial potential against a set of nine human pathogens strains was achieved. The obtained results showed the richness of the studied extracts in total phenolics, flavonoids and condensed tannins with significant variation among the studied populations.

The investigated extracts showed antibacterial and antifungal activity against all tested microbial strains with variable degree according to the microbial strain and the studied extract. The highest antibacterial activities were observed against the three gram-positive bacterial strains while the highest antifungal potentials were revealed against the two Aspergillus fungi species. It is clear that the genetic backgrounds influence significantly the accumulation of phenolic secondary metabolites in Artemisia arborescens and then their antimicrobial potentials. Consequently, the conservation of Artemisia arborescens genetic resources to maintain the highest chemical polymorphism of this endangered species in Tunisia is required. The obtained findings promoted this species as a candidate for further investigations for the development of natural antimicrobial agents with wide applications in cosmetic, food and pharmaceutical industries.

Keywords: *Artemisia arborescens* L., Phenolic compounds, Antimicrobial potential, Bioresources.

Introduction

Plant secondary metabolites play significant roles in maintaining human health through both preventive and therapeutic ways.[45,53] This class of biomolecules makes a part from wide range of plant natural products valorised and used since the ancient times as a reservoir of powerful and effective health promoting agents.[24] Secondary metabolites include a wide array of biomolecules such as phenolic acids, flavonoids, tannins and alkaloids among others which have showed their efficiency in sustaining both plant, human and animal health.[8,39] Recently, the application of multitudes of secondary metabolites compounds as ingredients and additives in cosmetic, food and pharmaceutical industries is increasing.[44]

Furthermore, an increasing number of scientific investigations evidenced broad spectrum of biological activities of phenolic secondary metabolites which can act as antioxidants, anticancer, anti-inflammatory, antidiabetic, anti-mutagenic and cardioprotective agents among others.[4,11,22,54] Furthermore, phenolic compounds were reported to highlight significant antiviral,[51] antibacterial and antifungal potentials and could be useful for microbial diseases control.[6]

Antibiotic-resistant pathogens continue to emerge worldwide and present one of the most serious global public health threats in this century and cause damage to human health and environment.[43] Thus, a multitude of microbial strains related to common or severe infections continue to emerge and to develop disturbing resistance to the available antibiotic drugs.[32] Plant natural bioresources are actually considered as a promising alternative in modern antibiotic drug discovery and development.[20] Some natural plant phenolics have shown their ability to act alone as antimicrobial agents or exert a synergistic effect in combination with common available antibiotics and enhanced their efficiency and thus present promising alternative to treat drug resistant microbial infections.[31]

The natural antimicrobial agents can be sustainably produced, are easily degradable and safer to human, animals and environment as compared to synthetic agents which give more advantages for their use and development.[25] Nowadays, synthetic products face a drastic decreasing of their acceptance by the consumers with a worldwide tendency for the benefit of natural products ingredients in modern pharmaceutical, cosmetic and food industries.[2,14]

Actually, these plant secondary metabolites have presented the basis for the discovery of several phytochemicals with wide application in the modern pharmaceutical industry.[27] The discovery of the antimalarial drug based on artemisinin

originated from the medicinal species *Artemisia annua* L. (*Asteraceae* family) presents the most famous example and was awarded Nobel Prize in 2015.[34] Thus, various species of the genus *Artemisia* known for their toxicity have given more attention for the screening of natural antimicrobial agents.[30]

Artemisia arborescens L. (*Asteraceae*), known as tree wormwood, is a medicinal and aromatic species endemic to the Mediterranean area.[19] This species is used in traditional medicine of these regions to treat a wide spectrum of metabolic disorders, respiratory diseases and infections.[5,7,29,38] The popular medicinal basis of tree wormwood is confirmed by various scientific reports which strongly indicated that the organic extracts of *A. arborescens* L. exhibited anti-inflammatory[5], pesticidal[12], phytotoxic[3], antiviral[44], anti-mycoplasma[1], nephroprotective and antioxidant properties.[17]

The biological actions of plant extracts are correlated with their richness in health promoting components among them phenolic secondary metabolites. The phenolic concentration in plant organs and tissues is strictly influenced by different endogenous and exogenous factors, among them the genetic and environmental factors. Based on the last considerations, this work aimed to characterise the variability of phenolic compounds concentration in hydro-ethanolic leaves extracts among four *Artemisia arborescens* L. populations originating from different geographical and climatic conditions of Tunisia. Furthermore, the *in vitro* antimicrobial activities of the studied extracts were individually tested against a set of nine human pathogens microbial strains including three gram positive bacteria, three gram negative bacteria and three fungal species.

Material and Methods

Plant material and extraction procedure: Fresh *Artemisia arborescens* L. leaves (Figure 1) were harvested at the flowering development stage of the species from four sites presenting different geographic and bioclimatic areas of Tunisia namely Menzel Bourguiba (AAr_MB), Ain Draham (AAr_AD), Bouficha (AAr_BO) and Fernana (AAr_FE). Per population, five mature plant individuals were randomly harvested from each site on June 2018. The characteristics of the studied populations were listed in table 1. The harvested plant leaves were washed with tap water, then air-dried in the shade at room temperature (25-30°C) for a week and then uniformly grinded using an electric grinder to be further used for extraction.

Table 1
Geographical and bioclimatic characteristics of the studied populations

Population	Code	Bioclimatic zone	Region of Tunisia
Menzel Bourguiba	AAr_MB	Sub Humid	North East
Ain Drahem	AAr_AD	Humid Superior	North West
Bouficha	AAr_BO	Semi Arid Inferior	North East
Fernana	AAr_FE	Sub Humid	North West

Figure 1: The studied species *Artemisia arborescens* L. (Asteraceae family)

Twenty grams of powdered dried leaves from each sample were subjected to a dynamic maceration with 100 mL of 80% (v/v) hydro-ethanol solution for 24 h in a rotary shaker at 30°C in the dark. Afterward, the obtained extracts were filtered using whatmann no.1 filter paper, then centrifuged at 2500 rpm for 10 min. The solvent was evaporated at 40°C using a rotary evaporator until having constant weight. The obtained residue was dissolved in bidistilled water and stored for further analyses. The extraction yields were calculated and expressed in percentages.

Total phenolic contents: The total phenolic contents (TPC) of ethanolic extracts were determined according to the Folin-Ciocalteu method [46] with some modifications. Briefly, 0.125 mL of adequately diluted extracts were transferred into a 2 mL Eppendorf tube and mixed with 0.625 mL of Folin-Ciocalteu reagent (10%). After 5 min, 0.250 mL of aqueous sodium carbonate solution (7.5%) was added. The mixtures were allowed to stand for 90 mn at room temperature (25°C) in the dark with intermittent shaking until the stabilisation of the colour, then the absorbance was measured at 760 nm. Gallic acid was used as standard to prepare a calibration curve (0-200 µg/mL) and the concentration of total phenolics was expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g DW).

Total flavonoid contents: The total flavonoid contents of the investigated extracts were achieved based on the aluminium chloride colorimetric following Chograni et al.[13] One millilitre of diluted extract was mixed with 1 mL of 2% $AlCl_3$ solution. After incubation for 15 min at room temperature in the dark, the absorbance was measured at 430 nm. Total flavonoid contents were calculated from the calibration curve using rutin (0-200 µg/mL) as standard and expressed as milligrams of rutin equivalent per gram of dry weight (mg CE/g DW).

Total condensed tannin contents: The total condensed tannin contents of *A. Arborescens* L. extracts were determined using the vanillin methods [47] with some modifications. A total of 750 µL of 4% vanillin solution and 375 µL of concentrated H_2SO_4 were added to 50 µL of adequately diluted extract. After 15 min, the absorbance of the mixture was measured at 500 nm. The contents of total condensed tannins were determined through a calibration curve using catechin (0-200 µg/mL) as standard. Results were expressed as milligrams of catechin equivalent per gram of dry weight (mg of CE/g of DW).

Antibacterial and antifungal activities: Nine human pathogens microbial strains were used to test individually the *in vitro* antibacterial and antifungal potential of tree-wormwood hydro-ethanolic extract. The antibacterial activities were evaluated against six human pathogenic bacterial strains representing three gram negative (*Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*) and three gram positive (*Staphylococcus aureus*, *Listeria monocytogenes* and *Bacillus cereus*) strains. The antifungal potential of these extracts was investigated against two fungi (*Aspergillus flavus*, *Aspergillus niger)* and one yeast (*Candida albicans*).

The antibacterial and antifungal activities of *A. arborescens* leaves extracts were determined by disk diffusion and broth dilution assays following Riahi et al.[40] Gentamicin and amphotricin B were used as a positive reference for bacteria and fungi respectively. Disc impregnated in solvent without sample was used as a negative control. The diameter of the inhibition zone diameter (ID) was measured in millimetres (including disk diameter of 6 mm) and achieved in triplicate. The minimum inhibitory concentration (MIC), the minimum bactericidal concentration (MBC) and minimum fungicide concentrations (MFC) values of *A. arborescens* leaves extracts against the nine microbial strains were determined based on broth dilution assays.

Data analysis: All experimentations were analyzed in three replications. The results were expressed as the means values and standard deviations. Statistical analysis was performed applying one-way analysis of variance (ANOVA) followed by Duncan's multiple range test at $p \leq 0.05$.

Results and Discussion

Extraction yields: Conventional extraction yields (% w/w) with reference to the air-dried plant material vary significantly between 7.47±0.45 (AAr_AD) to 12.33±0.51 % (AAr_BO) with a mean value of 9.95±2.01 % (Table 2). These findings highlighted the efficiently of 80% hydro-ethanol solvent in the extraction of phenolic compounds.

This result corroborates previous studies showing the efficiency of 80% hydro-ethanol solvent in conventional maceration method for the extraction of high value phenolic metabolites with considerable antimicrobial potential.[23,42] Ethanol and methanol are among the most frequent solvents used for the extraction of plant phenolic compounds.[13,52]

However, ethanol solvent is considered safer for human manipulation and consumption and is for these reasons preferred as a solvent in cosmetic, pharmaceutical and food industries.[49] Furthermore, ethanol can be sustainably produced from renewable biosources giving more advantageous to its use.[33]

Phytochemical characterisation: The leaves hydro-ethanolic extracts of Tunisian *Artemisia aroborescens* ecotypes were screened for their amounts of total phenolics, flavonoids and condensed tannins. Significant differences were recorded among the total phenolic amounts of the studies populations (Table 2). The highest total phenolic content (118.37±4.71 (mg GAE/g DW)) was recorded for the population of Bouficha AAr_BO (North East, Semi Arid Inferior) while the lowest levels (80.66±2.34 mg GAE/g DW) were obtained for the population of Ain Draham AAr_AD (North West, Humid Superior).

Table 2
Variability of extraction yields, total phenolic, flavonoid and condensed tannin contents among *A. arborescens* ethanolic extracts. (AAr_MB: Menzel Bourguiba, AAr_AD: Ain Draham, AAr_BO: Bouficha, AAr_FE: Fernana)

Population	Yield (%)	TPC (mg GAE/g DW)	TFC (mg RE/g DW)	TTC (mg CE/g DW)
AAr_MB	9.56±0.59b	96.59±3.68b	47.66±1.09b	25.14±0.6d
AAr_AD	7.47±0.45a	80.66±2.34a	38.17±1.17a	22.15±0.52c
AAr_BO	12.33±0.51c	118.37±4.71d	55.38±1.60c	16.03±0.37a
AAr_FE	10.44±0.37b	107.55±3.13c	53.11±1.02c	19.37±0.53b

The total flavonoid contents vary significantly among the four studied germplasms as the same way as total phenolic contents (Table 2) and range from 38.17±1.17 mg RE/g DW (AAr_AD) to 55.38±1.60 mg RE/g DW (AAr_BO). The variation of the amounts of condensed tannin contents did not exhibit the same pattern as total phenolic and flavonoid contents (Table 2).

The population of Menzel Bourguiba AAr_MB (North East, Sub Humid) possessed the highest level of condensed tannin contents (25.14±0.60 mg CE/g DW) while the population of Bouficha AAr_BO showed the lowest accumulation level (16.03±0.37 mg CE/g DW).

The obtained results highlighted the richness of Tunisian tree wormwood in phenolics. These findings valorise the studied species as a candidate for the sustainable production of high value secondary metabolites with various therapeutic abilities. Previous reports revealed the efficiency of phenolic secondary metabolites of *Artemsia* species as preventive and therapeutic agents.[35] Given wide range of biological activities, these classes of phenolic compounds recorded an increase interest as ingredients and additives in pharmaceutic, cosmetic and food industries.[10,21,37]

It is noted that the contents of these secondary metabolites vary significantly among the studied populations. The two populations AAr_BO and AAr_FE populations exhibited the highest concentration levels of total phenolic and flavonoid compounds in their leaves whereas the highest condensed tannin contents were observed for AAr_MB and AAr_FE *Artemisia arborescens* germplasms.

The last findings confirm previous investigations which highlighted the effect of genotypic and environmental conditions in the concentration of phenolic metabolites in plant tissues which resulted in a high intraspecific phytochemical variability.[36] Based on the last considerations, the urgent conservation strategies of the genetic resources of medicinal crops with high therapeutic potentials such as *Artemisia arborescens* through *in situ* and *ex situ* measures are required.

The maintenance of the highest chemical polymorphism of medicinal and aromatic species for which the secondary metabolites are the traits of interest for improvement and selection will provide more possibilities in varietal creation programs in the future.[41]

Antibacterial activities: The antibacterial potential of *A. arborescens* leaves extracts representing four different Tunisian germplasms was evaluated against three gram-positive and three gram-negative bacterial strains. The inhibition diameters zones (ID), the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were used as qualitative and quantitative parameters. The obtained results indicated that all *A. Arborescens* hydro-ethanolic extracts were shown effective against the six tested bacterial strains with variable degrees according to the plant extract and to the tested bacterial strain.

The overall data highlighted that the highest antibacterial potentials were observed against gram positive bacterial strains. The inhibition zone diameters vary from 13 mm to 19 mm for gram negative bacterial strains and from 18 mm to 25 mm for gram positive strains (Figure 2).

The inhibition zone diameters have shown significantly higher ID values than Gentamicin for the three gram positive strains with the AAr_MB and AAr_AD extracts. Comparable values to ID values of Gentamicin were recorded for the others two sites against the gram positive strains.

The observed sensitivity of the tested three gram negative bacteria while lower than for gram positive ones is still considerable based on the studied parameters. The minimum inhibitory concentrations (MIC) and the minimum bactericidal concentrations (MBC) confirm the results obtained for ID parameter and showed gram positive bacteria as the most sensitive to *Artemisia arborescens* extracts. The MIC/MBC values vary from 12.5/25 to 50/100 µg/mL for gram negative strains and from 6.25/12.5 to 25/50 µg/mL for gram positive bacteria (Table 3). The highest antibacterial activities against negative strains were recorded against *P. aeruginosa* with AAr_MB and AAr_AD ethanolic extracts (MIC/MBC: 12.5/25 µg/mL). It is noted that antibacterial activities vary depending both on the tested strain and the used extract.

The obtained results are in accordance with previous investigations which reported the antimicrobial activities of phenolic secondary metabolites through direct and indirect modes of actions.[15] Various studies provided the mechanisms of actions of the antibacterial activities of phenolic compounds and concluded that this is achieved

through a cascade of reactions including destabilization of bacterial membrane, disruption of the lipid bilayer, inhibition of enzymes and toxins, inhibition of ATP synthesis and inhibition of formation of bacterial biofilm.[31,50]

Antifungal activities: The *in vitro* antifungal activity of *A. arborescens* ethanolic extracts originating from four different Tunisian localities against three fungal species representing two fungi and one yeast strains were tested using the inhibition diameters zones (ID), the minimum inhibitory concentrations (MIC) and the minimum fungicidal concentrations (MFC). Based on the results of the disc diffusion assay, the four tested extracts exhibited antifungal activity against the three tested fungi and yeast species with ID values ranging from 12 mm to 17 mm (Figure 3).

It is noted that the highest inhibition zones diameters were observed against the two fungi species *A. flavus and A. niger* which highlighted higher ID values than the antifungal positive control Amphotericin B. The obtained results revealed that the MIC/MFC values ranged from 12.5/25 µg/mL to 100/200 µg/mL (Table 3). According to our results, the lowest MIC and MBC (12.5/25 µg/mL) values were recorded against *A. flavus* species with AAr-MB population extracts.

The obtained results showed an antifungal activity especially against the two fungi species *A. niger* and *A. flavus* which have been shown most sensitive to *A. arborescens* phenolic extracts than *Candida albicans*.

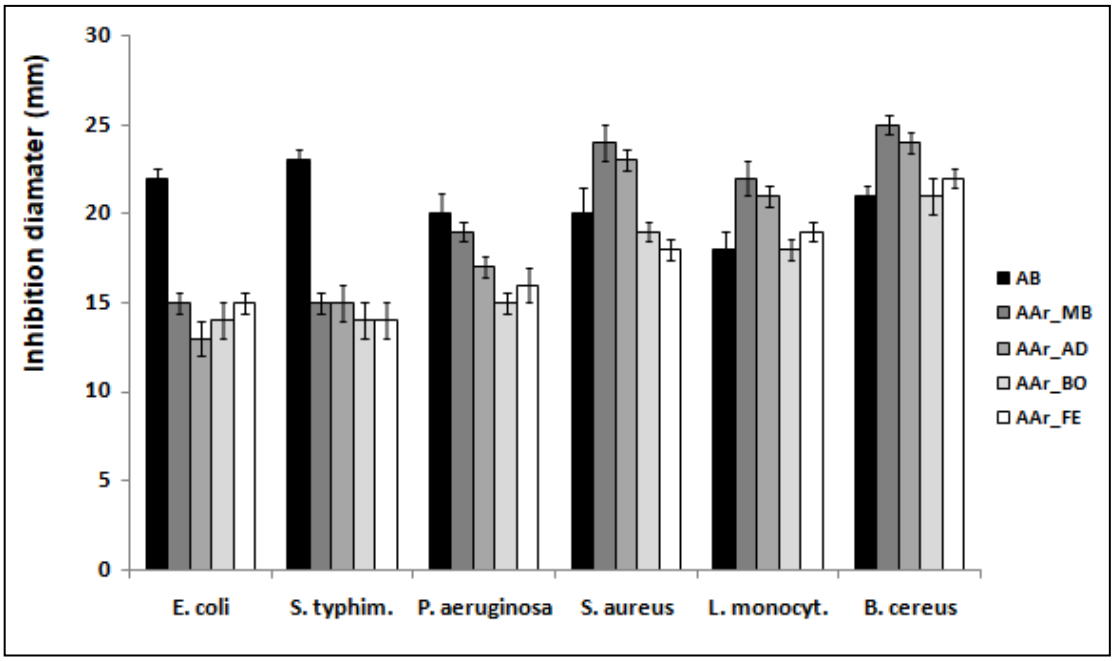

Figure 2: Variability of antibacterial inhibition zone diameters ID (mm) among the studied ethanolic extracts of *A. arborescens* L. (AAr_MB: Menzel Bourguiba, AAr_AD: Ain Draham, AAr_BO: Bouficha, AAr_FE: Fernana). AB: Gentamicin antibiotic.

Table 3
Variation of MIC and MBC and MFC (µg/mL) values of the studied extracts against nine pathogenic microbial strains. (AAr_MB: population Menzel Bourguiba, AAr_AD: population Ain Draham, AAr_BO: population Bouficha, AAr_FE: population Fernana)

	AAr_MB	AAr_AD	AAr_BO	AAr_FE
E. coli	25/50	50/100	25/50	25/50
S. typhimurium	25/50	25/50	25/50	25/50
P. aeruginosa	12.5/25	12.5/25	25/50	25/50
S. aureus	6.25/12.5	12.5/25	12.5/25	12.5/25
L. monocytogenes	12.5/25	6.25/12.5	12.5/25	12.5/25
B. cereus	6.25/12.5	6.25/12.5	12.5/25	6.25/12.5
A. flavus	25/50	12.5/25	25/50	25/50
A. niger	12.5/25	25/50	25/50	25/50
C. albicans	50/100	50/100	50/100	100/200

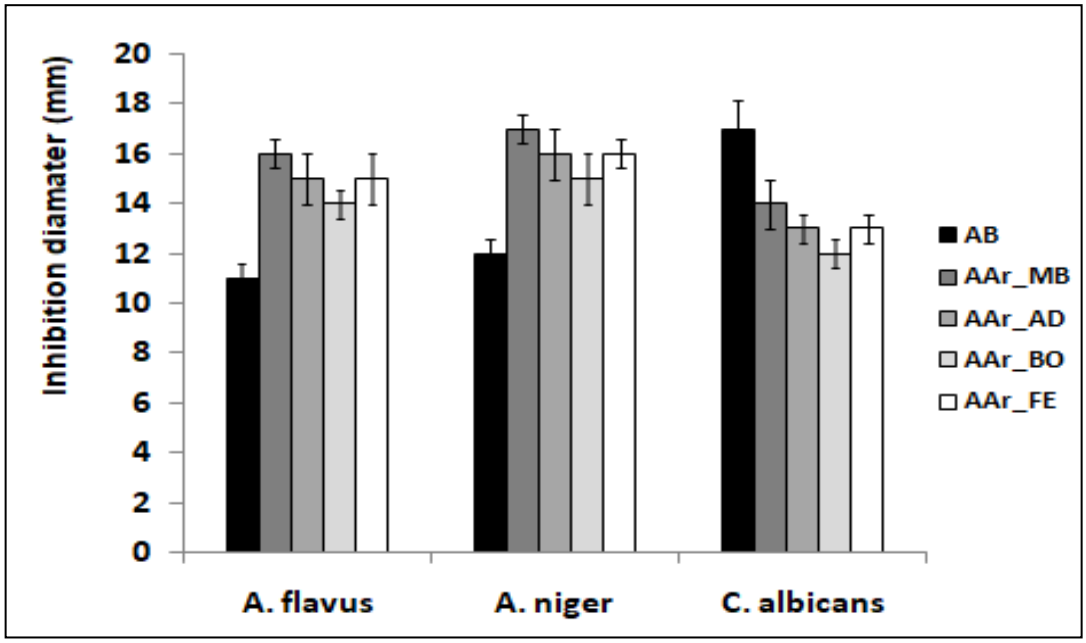

Figure 3: Variability of antifungal inhibition zone diameters ID (mm) among the studied ethanolic extracts of *A. arborescens* L. (AAr_MB: Menzel Bourguiba, AAr_AD: Ain Draham, AAr_BO: Bouficha, AAr_FE: Fernana). AB: Amphotericin B antibiotic.

Our results corroborate with findings by Elansary et al[18] who reported the efficiency of phenolic extract of *Ferocactus* species against *Aspergillus* species while *Candida albicans* species have been shown relatively resistant. *A. niger* and *A. flavus* are common infectious foodborne pathogens reported to produce the carcinogen aflatoxins and pose a significant threat to human and plant health.[9,16] *Aspergillus niger* and *Aspergillus flavus* cause contamination in the fruits and vegetables of various economic crop species and cause cancer in humans and animals.[26,28]

Actually, the reported cases of antifungal drugs resistance among *Aspergillus* species are increasing.[48] Interestingly, *Artemisia arborescens* phenolic extracts present a potential for the development and the sustainable production of antifungal agents.

Conclusion

The obtained findings revealed the richness of *Artemisia arborescens* L. in potential active pharmaceutical ingredients such as phenolics, flavonoids and condensed tannins. The contents of these high-value constituents vary significantly according to the studied population which requires the necessity of the conservation of the genetic resources of this species threatened of extinction in Tunisia for further commercial exploitation and breeding programs.

Moreover, the investigated hydroethanolic extracts exhibited considerable antibacterial and antifungal potential against the nine human pathogens microbial strains especially against the three gram positive bacteria and the two *Aspergillus* fungi species. This report supports the traditional medicinal use of this species in the treatment of infections and suggests tree wormwood as promising candidate for the development of natural antimicrobial agents.

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(Received 20th April 2020, accepted 22nd June 2020)