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Kombucha fermentation of African mustard (*Brassica tournefortii*) leaves: Chemical composition and bioactivity

Rami Rahmani^{a,b}, Sandra Beaufort^b, Silvia Alejandra Villarreal-Soto^b, Patricia Taillandier^b, Jalloul Bouajila^{b,**}, Mohamed Debouba^{a,*}

^a Unité de Recherche, Valorisation des Biomolécules Actives, Institut Supérieur de Biologie Appliquée de Médenine, Université de Gabés, Gabés, Tunisia

^b Laboratoire de Génie Chimique, Université de Toulouse, CNRS, INPT, UPS, Toulouse, France

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ABSTRACT

Brassica tournefortii is an edible vegetable formerly consumed by North African populations. Nowadays, this plant has been neglected and is less used. The present study aims to give an extra nutraceutical value to *B. tournefortii* using a 2-wk kombucha fermentation process. At the end of incubation, fermented and unfermented (control) *B. tournefortii* aqueous extracts were successively fractionated with ethyl acetate (EtOAc) and n-butanol to measure their chemical composition and bioactivity. Results showed that kombucha fermentation significantly increased total phenolic content, with the highest amounts in the EtOAc fraction. The antioxidant potential of *B. tournefortii* leaves was improved by fermentation of EtOAc extracts and conversely lowered in aqueous ones. Anti-acetylcholinesterase activity was increased with fermentation to reach ~8-fold higher value in *B. tournefortii* EtOAc and aqueous extracts relative to unfermented samples. Kombucha fermentation was found to reduce cytotoxicity and xanthine oxidase inhibitory effects of *B. tournefortii* leaves. The findings suggested that fermentation is a promising, simple and safe bioprocess that could improve the food properties of less-used edible plants.

1. Introduction

The genus *Brassica* includes many species of economic importance. These species can be the sources of seed oil (*B. rapa*, *B. oleracea*, *B. napus*), and also the sources of bioactive compounds such as glucosinolates, polyphenols, carotenoids and vitamins (Dal, Silva, Bolssoni, Antônio, & Viana, 2013). These bioactive compounds found in Brassicaceae crops are known for their involvement in human nutrition and they have several health benefits, including, antioxidant, anti-inflammatory, anti-microbial, anti-allergic, cytotoxic and anti-tumor activities (Cartea, Francisco, Soengas, & Velasco, 2011). The consumption of a diet good in *Brassica* vegetables was associated with inhibitions of chemically induced carcinogenesis in laboratory animals and humans (Ioana, Socaci, & Socaciu, 2012).

Brassica tournefortii (Brassicaceae), also called African mustard, is an annual herb plant growing wild in the Mediterranean regions of North Africa and the Middle East (Minnich & Sanders, 2000). It grows well in sandy soils and regions with eolian sediment (Sánchez-Flores, 2007). Little information is available about *B. tournefortii* chemical

composition and biological activities. Formerly, *B. tournefortii* was collected and used as a cooked legume within traditional meals. Preliminary work showed that *B. tournefortii* leaves accumulated low levels of secondary metabolite and had a weak bioactivity (unpublished data). Hence, in the present study, *B. tournefortii* samples were the subject of a short term kombucha fermentation that aimed at studying the potential production of new metabolites and the evaluation of bioactivities.

Kombucha is a refreshing drink obtained by fermentation, for about 14 d with a symbiotic culture of several indigenous bacteria (*Acetobacter* and *Gluconobacter*) and yeasts (*Saccharomyces* spp and *non-Saccharomyces* spp) (Malbasa et al., 2014). Most of the studies suggest that “tea fungus” came from the Southeast of Asia, Japan, Tibet or Manchuria and dates back thousands of years (Jarrell, Cal, & Bennett, 2000). A billowing cellulosic pellicle film and an acid liquid broth are the two portions which compose the “tea fungus”. It has been claimed that kombucha beverages are a prophylactic agent beneficial to health (Srihari & Satyanarayana, 2012; Villarreal-Soto, Beaufort, Bouajila, Souchard, & Taillandier, 2018). Nowadays, the preparation of kombucha is not limited to cultivation in sweetened black tea. Other

* Corresponding author. Université de Gabés, Institut Supérieur de Biologie Appliquée, Route El Jorf Km 22.5, 4100, Medenine, Tunisia.

** Corresponding author. Université Paul Sabatier, Faculté des Sciences Pharmaceutiques, Service de Chimie Analytique et Œnologie Coques A, 35 chemin des Maraîchers, 31062, Toulouse, France.

E-mail addresses: jalloul.bouajila@univ-tlse3.fr (J. Bouajila), mohamed.debouba@gmail.com (M. Debouba).

Abbreviations

AChE	acetylcholinesterase	MCF-7	human breast cancer cells
ACTHI	acetylthiocholine	MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide	n-BuOH	n-butanol
DMEM	Dulbecco's modified eagle medium	NIST	National Institute of Standards and Technology (Gaithersburg, MD, USA)
DMSO	dimethyl sulfoxide	SD	standard deviation
DPPH	1-1-diphenyl-2-picryl hydrazyl	THF	tetrahydrofuran
DTNB	5,5'-dithiobis-2-nitrobenzoic acid; dw: dry weight	TMCS	trimethylchlorosilane
EtOAc	ethyl acetate	TPC	total phenolic content
GAE	gallic acid equivalents	U	unit
GC-MS	gas chromatography-mass spectrometry	XOD	xanthine oxidase

substrates can be used instead of tea, such as: fruit drink, wine, milk, herbal teas, lemon balm tea and green tea. Some of these new substrates have been shown to better stimulate kombucha fermentation compared to the original kombucha tea (Vitas, Malbaša, Grahovac, & Lončar, 2013). Dried leaves from *B. tournfortii* were incubated for 2 wk with kombucha “tea fungus” using controlled conditions. At the end of the treatment, fermented extracts were compared to unfermented controls in terms of their (i) total phenolic content (TPC) (ii) antioxidant (DPPH assay), anti-acetylcholinesterase (AChE), anti-xanthine oxidase (XOD) and cytotoxic activity (MCF-7). Data obtained highlighted the fermentation-related changes of chemical composition and bioactivity of *B. tournfortii* leaves.

2. Materials and methods

2.1. Chemicals used

All chemicals used were of analytical reagent grade. All reagents were purchased from Sigma Aldrich (Aisne, France): ACTHI, acetylcholinesterase, DMSO, DPPH, DTNB, EtOAc, Folin Ciocalteu reagent (2N), gallic acid, HCL, KH_2PO_4 , MeOH, MTT, NaOH, Na_2HPO_4 , n-BuOH, sodium carbonate, tamoxifen, XOD.

2.2. Plant material

Samples of *B. tournfortii* leaves were obtained from plants growing spontaneously in their natural habitats. The material was authenticated by Dr Mohamed Tarhouni (expert in botany at the Range of Ecology Laboratory, Arid Land Institute in Medenine, Tunisia). Samples originating from the region of Zarzis located in the southeast of Tunisia (33°30'14" N; 11°06'43" E). The plants were harvested in the period between February and March 2017, corresponding to their full bloom period, and dried at room temperature (20–25 °C) in the research unit of the High Institute of Applied Biology (Medenine, Tunisia). After 15 d, the dried leaves were ground using a mixer (Moulinex AT 710131, Normandy, France), into fine powder and preserved, no more than two months, in dark glass containers until their fermentation.

2.3. Fermentation assay

The fermentation procedure was carried out at the Laboratory of Chemical Engineering (LGC) in the National Polytechnic Institute (INP) of Toulouse (France) using the method adapted from Jayabalan, Malbaša, Lončar, Vitas, and Sathishkumar (2014). Dry leaves of *B. tournfortii* were used as the raw material for the infusion. Sucrose (77 g) was dissolved in 1100 mL of tap water and then heated to 90 °C. Then, 10.5 g of leaves powder were put in a small cotton ham sock, resistant to boiling water, (Concept Epices SARL, Pays de la Loire, France), placed in boiling water and allowed to infuse for about 15 min. After that, the preparation was left to cool to 25–30 °C before the addition of 7 g of the kombucha strain, also called “Scoby”, obtained from

an old preparation of kombucha tea. The used “Scoby” was cultured in the same medium for 14 d. The sugar solution was divided into three portions of 350 mL that were separately placed into 1 L glass jars. Two portions of the preparation were inoculated with 2% (v/v) of the previously fermented liquid tea broth and 1% (v/v) of apple cider vinegar (50 grain) (Amora, Dijon, France). Every 2 d, the “Scoby” was watered by its own solution to prevent dehydration or excess moisture. The fermentation was carried out at 25 °C for 14 d.

2.3.1. Analysis of sugars, ethanol and acetic acid

Samples of 1 mL (put in Eppendorf tubes (were taken, from each glass jar, on 3, 7, 10, and 14 d of fermentation and stored at –80 °C for HPLC analysis, a maximum of 4 wk. At the end of the fermentation, samples were centrifuged at 7840 g force (10,000 rpm) for 5 min at 4 °C, using an Eppendorf centrifuge (Mini-centrifuge mySPIN 6, Thermo Fisher Scientific, Strasbourg, France). Then, the supernatant fluid was filtered through a membrane filter (Millex-HA filter 0.45 µm, Sigma Aldrich, France). The filtrate obtained was subjected to analysis of sugars, ethanol and acetic acid using HPLC (Ultimate 3000, Thermo Fisher Scientific, France). Ten µL of each filtrate was injected into the HPLC system equipped with a refractive index detector in series (RefractoMax 521, Thermo Fisher Scientific, Waltham, MA, USA), an UV detector (VWD-3100, Thermo Fisher Scientific, USA) and a Rezex ROA-Organic acid H+ (8%), 250 × 4.6 mm phase-reverse column (Phenomenex, Le Pecq, France) thermostated at 30 °C. The elution was done at a flow rate of 170 µL/min, using a mobile phase that consisted of 10 mM sulfuric acid solution (pH = 2.2). The concentrations of each compound were quantified using standard curves and expressed as g/L.

2.3.2. Fractionation of unfermented (infusion) and fermented beverages

The aqueous beverages obtained after infusion (350 mL) and fermentation (350 mL) were successively extracted with solvents of increasing polarity (EtOAc and n-BuOH) (1:1, v/v). The liquid-liquid extraction was done twice with 350 mL of each solvent (EtOAc then n-BuOH). The organic extracts (700 mL of each) and the remaining aqueous phase were concentrated using a rotary evaporator (RV 10 Auto V, IKA, Staufen, Germany) under a vacuum at 35 °C. The dry residues obtained were put in hemolysis tubes and stored for up to 2 yr at –20 °C. The three concentrated fractions; EtOAc, n-BuOH and aqueous fraction were tested for their chemical composition (TPC and GC-MS), antioxidant activity, and biological activities.

2.3.3. Determination of pH

The pH values of the samples were measured using an electronic pH meter (pH2700, Eutech, Thermo Fisher Scientific, USA).

2.3.4. Enumeration of the total yeasts counts

Samples of the fermentation liquor were extracted from each glass jar for yeast enumeration. After gentle mixing, samples of 1 mL (put in Eppendorf tubes (were taken on 3, 7, 10, and 14 d of fermentation. A Thoma cell counting chamber (Thomas Scientific, Swedesboro, NJ,

USA) was used. For microscope observations, a drop of the liquor with a cover slip was placed on the stage of a binocular microscope (Laborlux 12 microscope, Leitz, Midland, ONT, Canada). A magnification of 10 or 40x was used.

2.4. Antioxidant activity (DPPH assay)

Free radical scavenging capacities of samples were determined using a DPPH method as described by Bekir, Mars, Vicendo, Fterrich, and Bouajila (2013), with slight modification. In a 96-well microplate (Micro Well, Thermo Fisher Scientific, France), 20 μ L of the diluted plant extract (0.5 mg/mL) were added to 180 μ L of 0.2 mM methanolic DPPH solution and the mixture was allowed to stand. A microplate reader (Multiskan Go, F1-01620, Thermo Fisher Scientific, Vantaa, Finland) was used to measure the absorbance at 515 nm, the wavelength of maximum DPPH absorbance. The A_{sample} , after an incubation period of 25 min at room temperature (15–20 °C), was measured. The A_{blank} was measured without extract. DPPH inhibition was calculated as:

$$\% \text{ inhibition} = 100 \times (A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}}$$

2.5. Total phenolic content (TPC)

The TPC of samples was determined using a Folin-Ciocalteu method with modifications (Bekir et al., 2013). The reaction mixture containing 20 μ L of diluted plant extract (0.5 mg/mL) and 100 μ L of Folin Ciocalteu reagent (0.2 N) was left at room temperature for 5 min before adding 80 μ L of sodium carbonate (75 g/L in water). After 25 min of incubation at room temperature, the absorbance was measured at 765 nm. A standard calibration curve was obtained using gallic acid (0–115 mg/L). Results were expressed as mg of gallic acid equivalents (GAE)/g of dw.

2.6. Biological activities

2.6.1. Anti-xanthine oxidase activity (XOD)

The XOD activity using xanthine as the substrate was measured spectrophotometrically using the procedure of Kohoude et al. (2017). The substrate solution (1 mM) was prepared by dissolving xanthine in 25 mL of 0.1 mM sodium phosphate buffer (pH = 7.5). The xanthine oxidase enzymatic solution was prepared by diluting xanthine oxidase enzyme from cow's milk (Sigma Aldrich) to a final concentration of 0.1 U/mL. The assay mixture consisted of 50 μ L of diluted plant extract (0.2 mg/mL), 60 μ L of 70 mM sodium phosphate buffer (pH = 7.5) and 30 μ L of the enzymatic solution, giving a final extract concentration of 50 mg/L in each well of a 96-well microplate. After 25 min of incubation, 60 μ L of substrate solution was added and the absorbance was measured at 295 nm after 5 min. The A_{blank} was measured without extract. The XOD activity was expressed as percent inhibition of XOD enzyme, calculated as:

$$\% \text{ inhibition} = 100 \times (A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}}$$

2.6.2. Anti-acetylcholinesterase activity (AChE)

The AChE activity was determined using the Ellman colorimetric method as previously described by Kohoude et al. (2017). Briefly, 50 μ L of 0.1 mM sodium phosphate buffer (pH = 7.5), 125 μ L of DTNB, 25 μ L of diluted plant extract (0.5 mg/mL) and 25 μ L of enzyme solution were mixed and incubated for 15 min at 25 °C. Thereafter, 25 μ L of ACTHI was added. Then the final blend was incubated for 25 min at 25 °C and the absorbance was measured at 421 nm. The A_{blank} was measured without extract. The enzyme activity inhibition percentage was calculated as:

$$\% \text{ inhibition} = 100 \times (A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}}$$

2.6.3. Cytotoxic activity

Cytotoxicity of the samples was estimated using the MCF-7 cell line, ordered from the American Type Culture Collection (ATCC Co., Manassas, VA, USA), as described by Bekir et al. (2013) with some modifications. Cells were distributed into 96-well plates at 3×10^4 cells/well in 100 μ L, and then 100 μ L of culture medium (DMEM) (Advanced DMEM, Thermo Fisher Scientific, France), containing samples at various concentrations were added. Cell growth was estimated using the MTT assay. MTT is a water-soluble tetrazolium salt with a yellow coloration. Metabolically active cells are able to convert the dye to water-insoluble dark blue formazan by reductive cleavage of the tetrazolium ring. The extracts were re-solubilized in the DMSO followed by dilution in the buffer whereby the DMSO does not exceed 1%. Tamoxifen was used as a positive control. The A_{blank} was measured without extract. The cells activity inhibition percentage was calculated as:

$$\% \text{ inhibition} = 100 \times (A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}}$$

2.7. Chromatographic analysis

2.7.1. Gas chromatography-mass spectrometry (GC-MS)

The identification of volatile compounds from *B. tournefortii* extracts, before and after derivatization, used the procedures of Kohoude et al. (2017). GC-MS analyses was done on a Varian Saturn 2000 ion trap GC/MS with CP-3800 GC (Varian, Walnut Creek, CA, USA), fitted with a fused silica capillary DB-5MS column (5% phenylmethylpolysiloxane, 30×0.25 mm, film thickness 0.25 μ m) (J & W GC Columns, Agilent Technologies, Santa Clara, CA, USA). The column oven temperature program was as follows: 60 °C hold for 5 min, up to 270 °C at the rate of 15 °C/min and then 6 min isothermally at 270 °C. Thereafter, another gradient was used to 300 °C at 50 °C/min and finally a 300 °C hold for 4.5 min. For analysis purposes, the samples were dissolved in their respective solvents. One μ L was injected in the split mode ratio of 1:10. Helium was used as the carrier gas at 1 mL/min. The injector was at 200 °C. The mass spectrometer (MS) was adjusted for an emission current of 10 μ A and electron multiplier voltage between 1400 and 1500 V. Trap temperature was 250 °C and that of the transfer line was 270 °C. The mass scanning was from 40 to 650 amu.

The identification of the compounds was done using mass spectra comparison with those obtained in NIST08 (National Institute of Standards and Technology, <https://www.nist.gov/>), using AMDIS software (Automated Mass Spectral Deconvolution and Identification System, <https://chemdata.nist.gov/dokuwiki/doku.php?id=chemdata:amdis>). AMDIS is a software used for GC-MS data interpretation from NIST. The goal was to find the maximum similarity, in terms of spectra, between the compounds detected in extracts and those suggested by the NIST database. Finally, the retention index facilitated certain assignments.

2.7.1.1. Derivatization method. The derivatization method was that described by Kohoude et al. (2017), with some modifications. In a 2 mL vial, 150 μ L of 99% BSTFA + 1% TMCS was mixed with 1 mL of extracts (5 mg/mL in THF solvent). Afterward, the mixture was agitated for 30 s to increase the solubility. The reaction mixture was maintained at 40 °C for 15 min. Ten μ L of each derivative solution was injected into the GC-MS and analyzed as described in the previous section.

2.8. Statistical analysis

All measurements were carried out in quadruplicate. One way analysis of variance (ANOVA) was used for the significance calculation using the Statistical Package for the Social Sciences (SPSS) 20.1 (Version IBM. 20.0. 2004, San Francisco, CA, USA and J Guru.com, <http://www.jguru.com/>). Statistical differences between the solvents used in the study were estimated using Tukey's test. The linear

correlation coefficient (R^2) was evaluated to determine the relationship between the TPC and antioxidant or biological activities. Principal component analysis (PCA) was also done using XLSTAT (version 2014.5.03, Addinsoft, Pearson edition, Waltham, MA, USA) to visualize the discrimination between the different parameters. The confidence limits were set at $p \leq 0.05$.

3. Results and discussion

3.1. Fermentation assay

3.1.1. Yeasts growth and pH changes

After 10 d of incubation, the total yeasts count slightly increased from 10^7 to 10^8 cells/mL in the liquid (Fig. 1A). By the end of fermentation, the growth of yeasts remained constant at 10^8 cells/mL. This suggests that the yeasts were growing in the medium using sugar and vegetable matter (Watawana, Jayawardena, Gunawardhana, & Waisundara, 2015). Furthermore, the role of yeasts in kombucha fermentation is important because they are known to be the responsible for glucose and fructose production through sucrose hydrolysis. These products can be used by a large spectrum of microorganisms during kombucha fermentation (Sievers, Lanini, Weber, Schuler-Schmid, & Teuber, 1995). The pH values of *B. tournefortii* fermented beverage had a statistically significant decrease ($p \leq 0.05$) as compared to the unfermented control (pH = 7). A rapid decrease of beverage pH was

observed during the first wk, dropping to pH = 3 at the end of fermentation (Fig. 1A). The changes in pH obtained are similar to those found by Velicanski, Cvetkovic, and Markov (2013) who used *Lamiaceae* herbs as a substrate for kombucha fermentation in small bioreactors. In the same way, the decrease of pH value in kombucha “tea fungus” beverages was reported recently by Neffe-Skocińska, Sionek, and Ścibisz (2017). They observed the decrease of pH value from 3.07 to 2.77 during 10 d of fermentation at 25 °C.

Probably, the main cause of the pH lowering may be the production of some organic acids (such as acetic acid, glucuronic acid and gluconic acid) during the fermentation (Fig. 1A).

Several investigations supposed that the low pH had a lot of beneficial effects, such as the protection of phenolic compounds’ bioactivity and the safeness of the fermented beverages against pathogenic microorganisms (Chen & Liu, 2000; Lucera, Costa, Conte, & Del-Nobile, 2012).

3.1.2. Changes in sugars, ethanol and acetic acid, contents

After 3 d of fermentation, almost 2/3 of the sucrose was hydrolysed by yeasts. One wk later, the sucrose content in the medium was gradually decreased until the hydrolysis was completed by the end of fermentation (Fig. 1B). Therefore, fermentation rate can be obtained by the time taken for sucrose to be used up (Malbasa, Loncar, & Djuric, 2008). The yeast invertase enzymes convert the sucrose into glucose and fructose (Troy & Terra, 2014). These two products were

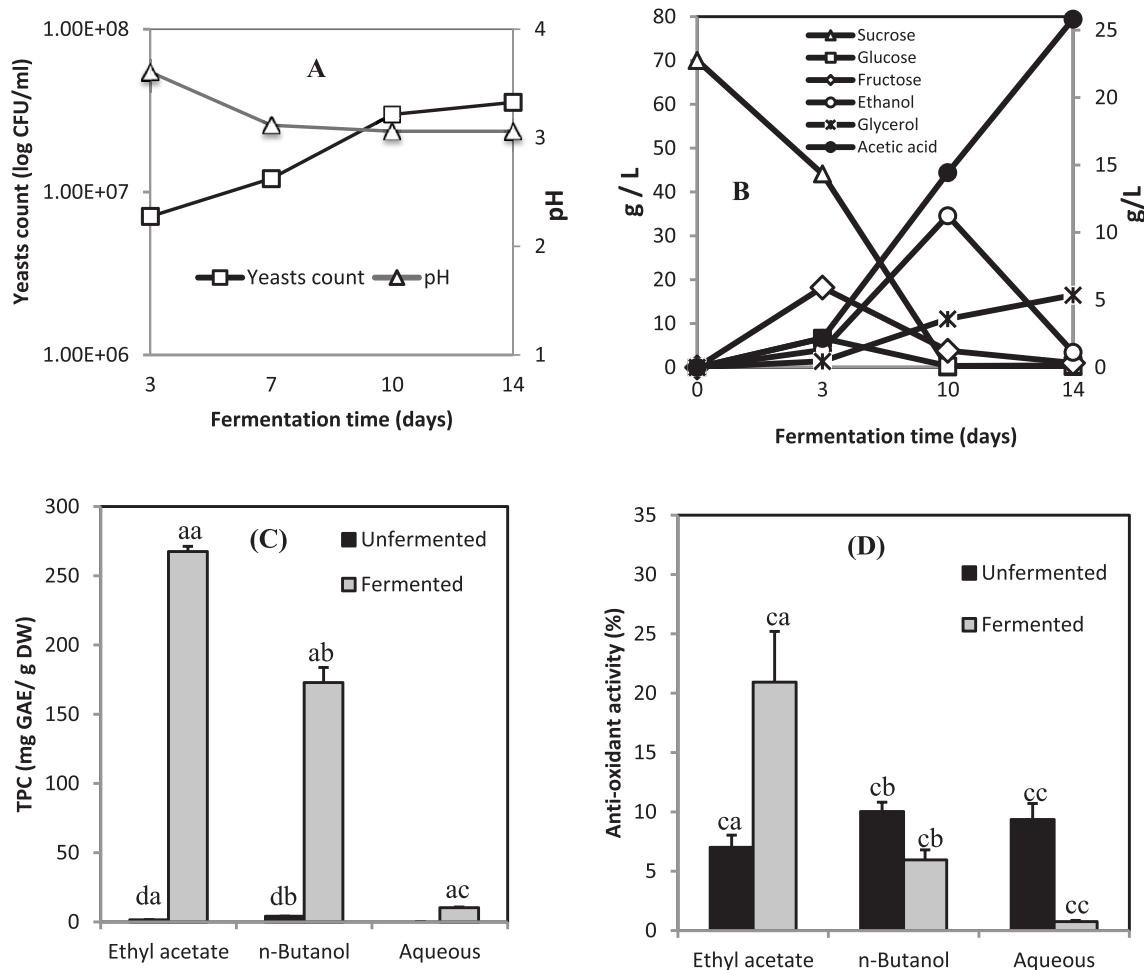


Fig. 1. Effects of kombucha fermentation of *Brassica tournefortii* leaves on (A) yeast growth and pH, (B) selected metabolites ((sucrose (left axis), glucose, fructose, ethanol and acetic acid (right axis)) concentrations, (C) total phenolic contents (TPC) and (D) DPPH radical scavenging activity. Unfermented infusion from *B. tournefortii* leaves using the same conditions were used as controls. Data are the mean of three repetition \pm SD. Different letters indicate significant differences according to Tukey test ($p \leq 0.05$).

accumulated in the medium, as observed by different authors during kombucha fermentation (Sievers et al., 1995; Chen & Liu, 2000; Kallel, Desseaux, Hamdi, Stocker, & Ajandouz, 2012). In the current study, glucose and fructose concentrations were concomitantly increased during the first 3 d of fermentation to reach 6.65 and 5.96 g/L, respectively (Fig. 1B). Nevertheless, other studies showed unequal concentrations between the two sugars, where fructose showed a higher concentration compared to glucose during a prolonged kombucha fermentation (up to 60 d) (Chen & Liu, 2000; Kallel et al., 2012). Here, the concentrations of fructose and glucose were less than expected concentrations normally obtained from the total sucrose hydrolysis (Fig. 1B). This showed that these two sugars were partially consumed by the different kombucha microorganisms. The remaining sugars was converted into ethanol, glycerol, and acetic acid or used in cellulose biosynthesis (Jayabalan et al., 2014; Sievers et al., 1995).

During the first wk of fermentation, the concentrations of ethanol and acetic acid both increased, from 0 to 11 and from 0 to 14 g/L, respectively (Fig. 1B). During the second wk, ethanol content decrease significantly to 1 g/L, while acetic acid increased to 26 g/L (Fig. 1B). In effect, acetic acid bacteria used the ethanol to produce acetic acid which is consider as the main product of kombucha fermentation and the principal reason for the pH decrease (Jayabalan et al., 2014). Actual results were consistent with previous works reporting changes in acetic acid and ethanol contents during “tea fungus” fermentation (Abbott & Ingledew, 2004; Sievers et al., 1995; Yang, Wang, Zhang, Tang, & Mao, 2016).

3.2. Total phenolic content (TPC)

A statistically significant increase in the TPC ($p \leq 0.05$) was observed in fermented *B. tournefortii* leaves for all the extracts, as compared to the unfermented control samples (Fig. 1C). The highest TPC was obtained for EtOAc (270 mg GAE/g dw) and n-BuOH (175 mg GAE/g dw) fractions. Differential contents of total phenols among solvents suggested that fermentation of *B. tournefortii* leaves led to secondary metabolites of different polarity and most of them were soluble in EtOAc (Fig. 1C). Quantitatively, TPC for fermented *B. tournefortii* leaves was greater than that reported in methanolic extracts of *Brassica napus* leaves (El-Beltagi & Mohamed, 2010). The fermentation increased TPC could be related to biotransformation of primary

metabolites present in a *B. tournefortii* leaves (aromatic amino acids, shikimic acids) by existing bacteria and fungi in kombucha (Watawana et al., 2015). A previous study showed that TPC of beverages obtained by the fermentation of sweetened lemon balm was higher than that of traditional kombucha fermentation with black tea (Velicanski, Cvetkovic, Markov, Tumbas-Saponjac, & Vulic, 2014). Furthermore, Yang et al. (2018) showed with vegetables-fruits fermented beverages that the fermentation increased the TPC content, which was ~1.25 fold higher for fermented than unfermented ones. However, others reported that fermentation decreased TPC, suggesting a possible biodegradation of polyphenols by yeast and bacterial enzymes during kombucha fermentation (Jayabalan, Marimuthu, & Swaminathan, 2007).

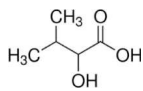
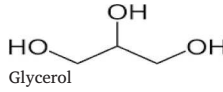
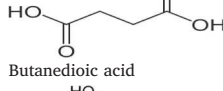
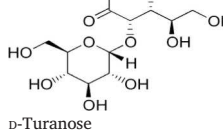
3.3. Antioxidant capacity (DPPH)

The antioxidant activity values measured as the extract capacity to reduce the free DPPH radicals are shown in Fig. 1D. Statistically, there was no significant increase ($p > 0.05$) in the antioxidant activity of the fermented samples compared to the unfermented control ones. All *B. tournefortii* extracts showed a low ability to neutralize DPPH free radicals. The unfermented samples antioxidant activity did not exceed 10%, while fermentation brought about 25% values for EtOAc extract (Fig. 1D). Similar antioxidant patterns were reported for *B. oleracea* (Podsedek, 2007). The influence of kombucha fermentation on antioxidant activity depends on two parameters; chemical and microbiological composition of the “tea fungus” on the one hand and the biochemical composition of the tested plant material on the other hand. For example, red bean fermented using *Bacillus subtilis* showed a low DPPH activity, but better Fe^{2+} -chelating ability (Chung, Chang, Chao, Lin, & Chou, 2002). Generally, the reducing power of beverages varies according to fermentation time. Chakravorty et al. (2016) found that the DPPH scavenging ratio of kombucha tea increased gradually from 3.7 to 44% in 3 wk of fermentation. Nevertheless, the recent study of Amarasinghe, Weerakkody, and Waisundara (2018) reported that the antioxidant activity of kombucha “tea fungus” decreased significantly during extended periods of fermentation (8 wk).

There was a positive modest correlation between the TPC and their respective antioxidant activity (DPPH) ($R^2 = 0.42$) (Fig. 1C and D). However, for *B. oleracea* extracts a strong positive correlation between DPPH and TPC was reported (Gawlik-Dziki, 2008; Kaur & Kapoor,

Table 1

GC-MS compounds detection and identification in fermented and unfermented leaves beverages of *Brassica tournefortii* after silylation.

N°	RT (min)	Compound	Unfermented			Fermented		
			H ₂ O	n-BuOH	EtOAc	H ₂ O	n-BuOH	EtOAc
1	10.18	 2-Hydroxy-3-methylbutyric acid	nd	nd	nd	nd	nd	X
2	11.33	 Glycerol	nd	X	nd	nd	X	X
3	11.90	 Butanedioic acid	nd	nd	nd	nd	X	X
4	20.52	 D-Turanose	nd	X	nd	nd	nd	nd

nd: not detected; H₂O: aqueous fraction; n-BuOH: n butanol; EtOAc: ethyl acetate.

2002). Here, the different patterns between TPC levels and antioxidant activity in *B. tournefortii* samples suggest that other antioxidant molecules (vitamin C, vitamin A) were probably efficient in reducing free radicals.

3.4. GC-MS analysis

Gas chromatography coupled with mass spectrometry was used to identify the volatile compounds in unfermented and fermented extracts of *B. tournefortii* leaves. No volatile compounds were detected without derivation.

Trying to identify more volatile compounds in the different extracts, a derivatization reaction was used to generate silylated products with chromatographic properties and volatility. No derivatized compounds were observed in fermented or unfermented aqueous extracts. On the other hand, this step led to the identification of 4 compounds in three extracts: unfermented n-BuOH, fermented EtOAc and n-BuOH extracts (Table 1).

The volatile profile from the different extracts showed the presence of two organic compound classes; acids and alcohols. Glycerol was detected using GC-MS in three fermented extracts: unfermented n-BuOH, fermented n-BuOH and fermented EtOAc. Butanedioic acid was detected in fermented n-BuOH and fermented EtOAc extracts, while 2-hydroxy-3-methylbutyric acid and D-turanose were found in fermented EtOAc and unfermented n-BuOH extracts, respectively (Table 1). GC-MS analysis showed a chemical composition difference between unfermented and fermented beverages of *B. tournefortii*. Except for glycerol, all other compounds were either in fermented extracts or in unfermented ones. Glycerol is supposed to be a metabolite product of kombucha fermentation (Kaczmarczyk, Zastosowanie, & Lochyński, 2014). However, the previous study of Vázquez-Cabral et al. (2017) indicated, similar to the present study, the presence of glycerol in unfermented extracts of oak leaves (*Quercus resinosa*).

The recent study of Ebrahimi, Mofid, Mofidi, Keyghobadi, and Ebrahimi (2017) showed that fermentation increased the number of chemical compounds and changed the chemical composition of

fermented beverages of garlic (*Allium sativum*).

3.5. Biological activities

3.5.1. Anti-acetylcholinesterase (AChE) activity

The AChE activity was determined photometrically by evaluating the extract's ability to inhibit AChE, which is the principal enzyme involved in the hydrolysis of acetylthiocholine (Giinther & Bilitewsk, 1995).

The anti-AChE activity of *B. tournefortii* leaves has not been studied previously. The unfermented control extract showed very low or no AChE inhibitory activity (Fig. 2A). Nevertheless, fermentation significantly improved the anti-AChE activity in EtOAc (37.7%) and aqueous (17.2%) extracts (Fig. 2A). The present results were within the range of anti-AChE values found in the literature for other Brassicaceae, such as red cabbage (*B. oleracea*) (Bo & Kolak, 2011; Ferreres et al., 2009). Results suggested that fermentation may generate new active metabolites against AChE in the EtOAc and aqueous extracts. Some reports, suggested a higher correlation between TPC and AChE inhibition activity (Amessis-Ouchemoukh et al., 2014; Papandreou et al., 2009). Whereas, in this study there was a significant modest correlation ($R^2 = 0.45$) between TPC and anti-AChE activity for both fermented and the unfermented *B. tournefortii* leaves (Figs. 1C and 2A).

3.5.2. Xanthine oxidase (XOD) inhibitory activity

XOD is a flavoprotein that catalyzes the oxidation of hypoxanthine to uric acid with xanthine as an intermediary. Therefore, XOD activity was evaluated as the formation of uric acid from xanthine (Sahgal et al., 2009). A significant difference ($p \leq 0.05$) was observed between the unfermented control and the fermented beverage (Fig. 2B). Based on the literature, fermented beverages usually showed strong XOD inhibitory activity (Jayabalan, Subathradevi, Marimuthu, Sathishkumar, & Swaminathan, 2008). Lee et al. (2010) found that a fermented sea tangle solution showed strong XOD inhibition. However, the current results showed that despite fermentation increased XOD inhibitory activity in EtOAc extract, the n-BuOH and aqueous extracts had less XOD

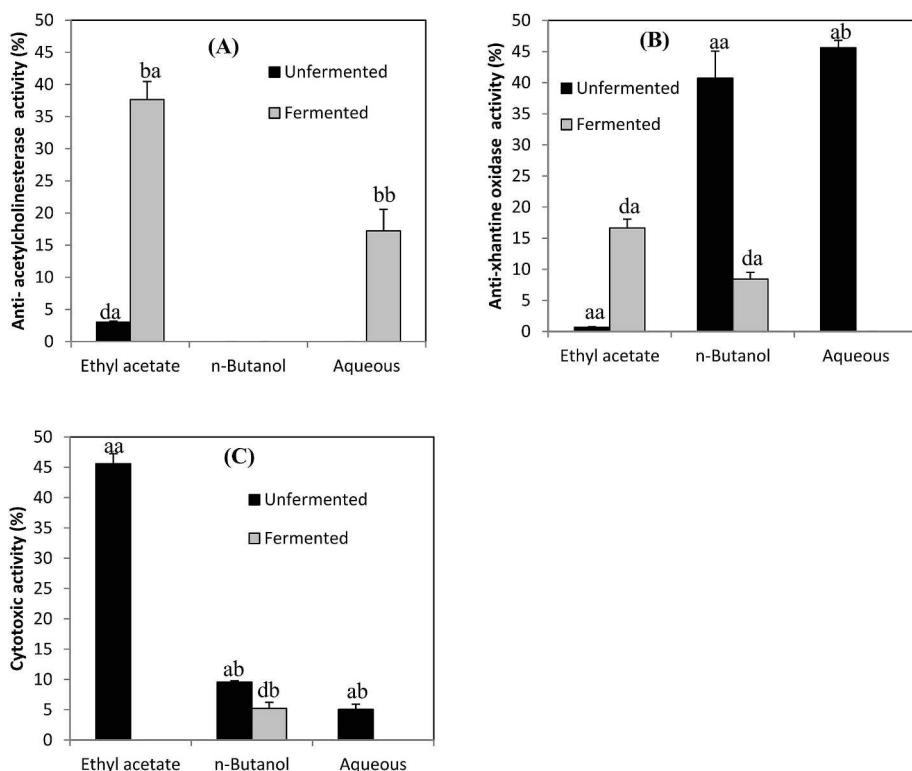


Fig. 2. Effects of Kombucha fermentation of *Brassica tournefortii* leaves on (A) anti-acetylcholinesterase (AChE) (B) anti-xanthine oxidase (XOD) and (C) cytotoxic activity (MCF-7) after 2 wk of fermentation process. Unfermented infusion from *B. tournefortii* leaves using the same conditions were used as controls. Data are the mean of three repetition \pm SD. Different letters indicate significant differences according to Tukey test ($p \leq 0.05$).

Table 2
Contribution of variable factors to the principal components analysis (%).

	F1	F2
Total phenolic content (TPC)	32.2	2.9
Radical scavenging activity (DPPH)	26.1	5.2
Anti-acetylcholinesterase activity (AChE)	29.8	7.0
Anti-xanthine oxidase activity (XOD)	0.0	71.8
Cytotoxic activity (MCF-7)	11.9	13.1

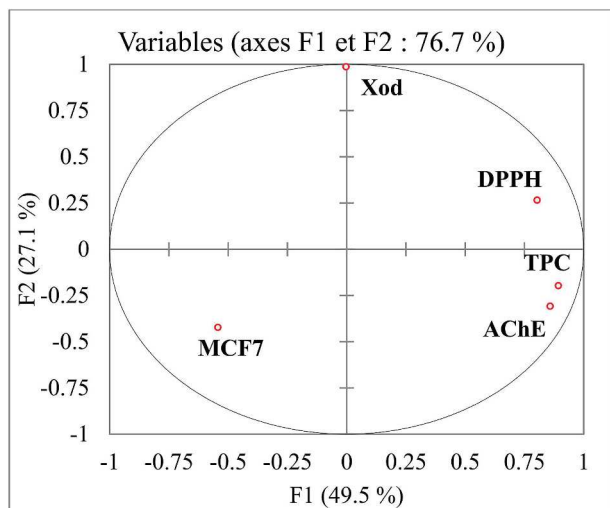


Fig. 3. Principal components analysis loading plot of antioxidant and biological activities of *B. tournefortii* beverages (unfermented and fermented). XOD: anti-xanthine oxidase activity; TPC: total phenolic content; AChE: anti-acetylcholinesterase activity; DPPH: antioxidant activity; MCF-7: cytotoxicity.

Table 3
Correlations between variables and factors.

	F1	F2
Total phenolic content (TPC)	0.89	-0.20
Radical scavenging activity (DPPH)	0.80	0.26
Anti-acetylcholinesterase activity (AChE)	0.86	-0.30
Anti-xanthine oxidase activity (XOD)	-0.01	0.99
Cytotoxic activity (MCF-7)	-0.54	-0.42

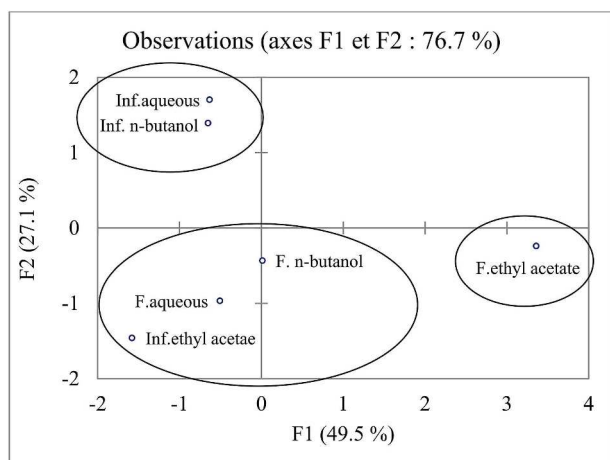


Fig. 4. Principal components analysis score plot of different *B. tournefortii* beverages (Inf: Infusion; F: Fermented).

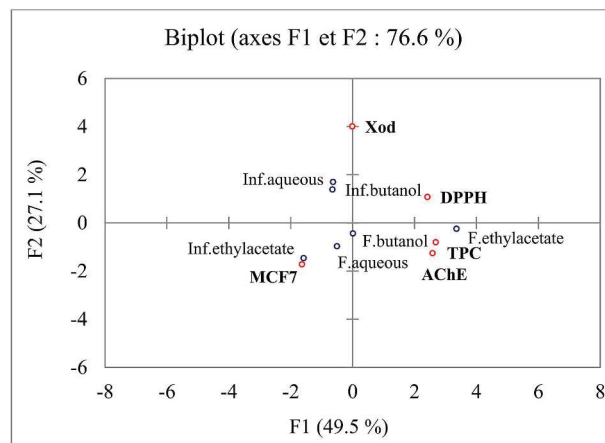


Fig. 5. Principal components analysis biplot of antioxidant and biological activities of different beverages of *B. tournefortii*. (Inf: Infusion; F: Fermented).

inhibitory activity (Fig. 2B). Anti-XOD activity in aqueous extracts was totally lost upon fermentation (Fig. 2B).

3.5.3. Cytotoxic activity

Cytotoxic activity of *B. tournefortii* beverage throughout fermentation against human breast cancer cells MCF-7, using an MTT assay, was evaluated (Fig. 2C). There was a high significant difference ($p \leq 0.05$) between unfermented and fermented beverages in terms of MCF-7 cell line inhibition. The highest MCF-7 cell growth inhibition was obtained for the unfermented EtOAc extract. This extract showed almost 45% inhibition (Fig. 2C). In a previous study, Cetojevic-Simin, Bogdanovic, Cvetkovic, and Velicanski (2008) showed that there is no difference between traditional kombucha and winter savory tea against MCF-7 cell line activity, and both of these kombucha showed about 15% inhibition. In this research, fermentation hardly reduced the EtOAc and aqueous cytotoxicity against MCF-7 cancer cells (Fig. 2C).

3.6. Principal components analysis (PCA)

Antioxidant and biological activities measurements of *B. tournefortii* beverages before and after kombucha fermentation have been analyzed using PCA. From this analysis, the axes of inertia had been withheld, as seen in Table 2. The structuring of accessions showed 76.7% of the total variation (Fig. 3). Axes were retained because they expressed 49.5 (PC 1) and 27.1% (PC 2). The loadings in the PCA loading plot express, at the same time, how well the principal components correlate with the original variables, and also the correlations between the different activities and TPC. PC 1 correlated well with anti-AChE activity, DPPH radical scavenging activity and TPC with loading of 0.86, 0.80 and 0.89, respectively. The second axis only reflected an anti-XOD loading of 0.99 (Table 3). Fig. 3 showed a good correlation between anti-AChE activity, DPPH radical scavenging activity and TPC, which suggested that the two activities were related, mainly through the TPC. Figs. 3 and 4 show the plots of the correlation loading and the factor scores. When applying principal component analysis, it seemed that there was a discriminate structure. Oval forms shown in Fig. 4 grouped the different beverages (unfermented, fermented) fractions in three classes. If the two plots (biplot) were gathering together, it is seen that the high TPC is related to the fermented EtOAc fraction and the unfermented aqueous phase fraction showed the highest cytotoxic activity (Fig. 5).

4. Conclusion

B. tournefortii dried leaves could be used as an alternative substrate to produce kombucha. Phytochemical analysis of fermented and unfermented (infusion) samples of *B. tournefortii* showed that (i) fermentation increased total phenolic compounds that were mostly extracted in the EtOAc fraction (ii) the use of a kombucha SCOBY inoculum with *B. tournefortii* infusion did seem to follow the same overall fermentation process as classical tea kombucha (iii) antioxidant and anti-AChE activities were improved with fermentation for the EtOAc fraction, (iiii) fermentation was found to decrease anti-XOD and cytotoxic action of *B. tournefortii* leaves. In addition, as the kinetics of sucrose, ethanol and acid acetic are close to those observed with kombucha “tea fungus”, it is possible to say that the activity of the microbial consortium does not seem to be disturbed by the change of support plant. In other words, the initial Scoby (preserved on tea infusion) has adapted very well to this new environment. Therefore, fermented *B. tournefortii* beverages could be an alternative natural healthy food. Further work regarding fermentation process optimization could improve the fermented *B. tournefortii* nutraceutical properties. Further work is in progress to identify the compounds formed after fermentation (HPLC-DAD and HPLC-MS) to explain the transformation mechanisms and the possibility of the formation of new compounds structures.

Conflicts of interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fbio.2019.100414>.

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