Preparation, chemical constituents of Podocarpus Nagi kernel oil and its in vitro antioxidant and anticancer activities

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November 11, 2020

Abstract

The Podocarpus Nagi kernel oil was prepared and its chemical constituents were confirmed accordingly in this work. The constituents and the contents of the obtained oil were identified as: total flavonoids (4.2%, calculated according to the rutin), (9Z,12Z)-9,12-octadecadienoic acid(38.2%), arachidic acid(0.11%), cis-11-eicosenoic acid(1.40%), cis-11,14-eicosadienoic acid(8.04%), -3(0.2%), -6(48.0%), -9(21.0%), vitamin E(2.11mg/100g). The microelements in this oil was also tested using ICP-MS and the results exhibited that this oil contains numbers of microelements beneficial for human health: such as V(0.006μg/g), Cr(0.024μg/g), Mn(40.109μg/g), Fe(2.292μg/g), Co(0.007μg/g), Zn(4.316μg/g), As(0.009μg/g), Se(0.240μg/g), Sr(0.453μg/g). Then, the oil was evaluated for its in vitro antioxidant effect against DPPH[?], which exhibited noteworthy scavenging ability against DPPH[?]. In addition, the oil was also evaluated for its preliminary in vitro anticancer activity against four cancer cell lines: the results showed that it exhibited the highest inhibition against gastric cancer, breast cancer (MCF-7), lung cancer(A549) and Hela cell lines with the highest inhibitions of 64.30%±2.80, 52.87%±2.57, 93.21%±2.24 and 88.33%±2.08 at the concentrations of 25mg/mL, 25mg/mL, 50mg/mL and 50mg/mL, and with the IC50s of 48.47mg/mL, 763.14mg/mL, 11.47mg/mL and 23.77mg/mL respectively. These findings demonstrated that this oil can be regarded as the functional edible oil by comparing the contents of edible oils used in the field of food.

Preparation, chemical constituents of *Podocarpus Nagi* kernel oil and its *in vitro* antioxidant and anticancer activities

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Abstract : The *Podocarpus Nagi* kernel oil was prepared and its chemical constituents were confirmed accordingly in this work. The constituents and the contents of the obtained oil were identified as: total flavonoids (4.2%, calculated according to the rutin), (9*Z* ,12*Z*)-9,12-octadecadienoic acid(38.2%), arachidic acid(0.11%), *cis* -11-eicosenoic acid(1.40%),*cis* -11,14-eicosadienoic acid(8.04%), -3(0.2%), -6(48.0%), -9(21.0%), vitamin E(2.11mg/100g). The microelements in this oil was also tested using ICP-MS and the results exhibited that this oil contains numbers of microelements beneficial for human health:

such as V(0.006μg/g), Cr(0.024μg/g), Mn(40.109μg/g), Fe(2.292μg/g), Co(0.007μg/g), Zn(4.316μg/g), As(0.009μg/g), Se(0.240μg/g), Sr(0.453μg/g). Then, the oil was evaluated for its *in vitro*antioxidant effect against DPPH[?], which exhibited noteworthy scavenging ability against DPPH[?]. In addition, the oil was also evaluated for its preliminary *in vitro* anticancer activity against four cancer cell lines: the results showed that it exhibited the highest inhibition against gastric cancer, breast cancer (MCF-7), lung cancer(A549) and Hela cell lines with the highest inhibitions of 64.30%±2.80, 52.87%±2.57, 93.21%±2.24 and 88.33%±2.08 at the concentrations of 25mg/mL, 25mg/mL, 50mg/mL and 50mg/mL, and with the IC_{50}s of 48.47mg/mL, 763.14mg/mL, 11.47mg/mL and 23.77mg/mL respectively. These findings demonstrated that this oil can be regarded as the functional edible oil by comparing the contents of edible oils used in the field of food.

Key words : *Podocarpus Nagi* kernel oil; Preparation; Chemical constituents; Antioxidant activity; Anticancer activity

Introduction

According to the statistics provided by the National Grain and Oil Information Center, since 1996, China has imported more edible oils than the total domestic production, and become the largest importer of edible oils and oilseeds in the world. The self-sufficiency rate of edible oils in China is currently less than 40%. In China, the oilseeds mainly rely on rapeseeds, peanuts, flaxseeds etc., which makes it still difficult to ensure the safe supply of the oilseeds and edible oils. In 2011, the consumption of edible oils reached 25.15 million tons, while industrial and other consumption reached 2.5 million tons, totaling 27.65 million tons, and the annual consumption per person has reached 20.5 kg in China. Among the edible oils, the import dependence of high-grade woody (herbaceous) edible oil has reached 95%, and the external dependence of ordinary edible oils has also reached 68%. Thus, it is urgent to explore new approaches and new raw oil materials to develop the edible oils for long-term development.

Podocarpus Nagi (*P. nagi* , named Zhubai in Chinese), belonging to the Podocarpaceae family. This plant contains different kinds of biological components (such as volatile oil, flavonoids, steroids, sugar and glycosides, lactones, etc.) and it exhibits a wide spectrum of biological activities: such as hemostasis, bone setting, anti-bacterial, anti-tumor, antiviral, antioxidant and detumescence activities [1-4]. The peel and fruit of *P. nagi*are rich in volatile components and oil, and especially the kernel of*P. nagi* has an oil content up to about 30%. *P. nagi* has many kinds of active components such as abundant unsaturated fatty acids, flavonoids, β-vanillin, vitamin E and so on [5]. Therefore, the *P. nagi* kernel oil should be a high-quality functional edible oil in theory. And according to the folk records of the Yao Nationality, *P. nagi* kernel oil has ever been used as an edible oil [6]. However, up to now, *P. nagi*kernel oil has not been used as an edible oil in the field of food yet. Considering the lack of edible oils in our native state and large scale of *P. nagi* kernel is produced ever year, it is very necessary to study the chemical constituents and relevant biological activities of*P. nagi* kernel oil to develop it to the fields of food and nutrition. In this work, the preparation process, chemical constituents and relevant biological activities (antioxidant and anticancer activities) of *P. nagi* kernel oil were studied.

2. Experimental

2.1 Material and instruments

The *P. nagi* kernel were collected in September of 2018 from the Yangli town of Fujian province and which were air-dried and powdered; other chemicals used for chemical extractions, chemical constituents analysis and biological evaluations are analytical reagents and commercially available.

Instruments. ICP-MS was carried out on the Agilent Technologies 7800(USA); Microwave digestion instrument (Mars 6 Classic, China); Full-wavelength multifunctional microplate reader (Multiskan GO, USA); Energy-saving water bath steamer (RQ-ZBL-12, China); Yifeng extruding machine (6Y1.0, China).

2.2 General process of oil extraction

The oil was obtained according to the below processes. Briefly, 50kg powdered *P. nagi* kernel was placed on a steamer with the mechanical stirring for 20min. Then, the steamed powder was transferred to a fixed mold with the high pressure device, and squeezed through pressure-programmed route from 0MPa to 30MPa for 30min to obtain the light yellow oil, 10kg, yield:20%.

2.3 Chemical components analysis of the obtained oil

2.3.1 Organic chemical components analysis

The organic components of the obtained oil were analyzed accordingly. The total flavonoids were determined in accordance with the《Implementation Manual of Technical Specifications for Health Food Inspection and Evaluation》(2003 Edition) P1082 [Determination of Total Flavonoids (Spectrophotometry)]; the fatty acids were determined according to the method of GB 5009.168-2016; and vitamin E was determined according to the method of GB 5009.82-2016.

2.3.2 Trace elements analysis

2.3.2.1 Prepare the testing solution

0.5g oil together with 10 mL of 65% nitric acid was added into the polytetrafluoroethylene (PTFE) digestion tank and the tank was sealed. The mixture was digested according to the following working conditions (see to **table 1** ; while the conditions of the ICP-MS instrument see to **table 2**). After completion of the digestion, the tank was cooled naturally to room temperature and the digestion solution was transferred to a 50 mL volumetric flask, and the tank was washed with a small amount of deionized water for three times and the washing solutions were also added to the volumetric flask. The volumetric flask was shaken well after filling to the constant volume with the deionized water to obtain the testing solution. The blank solution was prepared according to the above method.

Table 1. Conditions of microwave digestion

Power(W)	Time(min)	Temperature(°C)	Holding time (min)
800	10	120	5
800	5	150	5
1600	5	180	20

Table 2. Conditions of the ICP-MS instrument

Testing items	Working conditions
RF power	1.50KW
Cooling gas flow (argon)	15L/min
Auxiliary gas flow (argon)	1.0 L/min
Atomized gas flow (argon)	1.0 L/min
Number of repetitions	3
Collision pool mode	KED
Peristaltic pump speed	0.3rps

2.3.2.2 Prepare the standard working stock solution

2.3.2.2.1 Preparation of standard working stock solution

0.1g of the mixed standard solution of 10 kinds of mixed elements and 0.1g of the nitrogen standard of Se element were respectively accurately weighed, and diluted to 10 mL with 2% nitric acid to form the mixed standard stock solution (1000 ng/mL) containing 11 metal elements.

2.3.2.2.2 Preparation of series of standard stocking solution

Appropriate amount of the above standard working stock solution was weighed accurately, and which was diluted with 2% nitric acid to a standard series of mixed solutions containing 0.2ng, 1ng, 5ng, 10ng, 50ng, 100ng and 500ng of each element per 1mL.

2.3.2.2.3 Preparation of internal standard working solution

Appropriate amount of the mixed internal standard solution was accurately weighed and diluted with 2% nitric acid to 50 ng/mL.

2.3 *In vitro* biological evaluation

2.3.1 *In vitro* antioxidant effect of the *P. nagi* kernel oil

The scavenging ability of *P. nagi* kernel oil to DPPH[?] was carried out in this work. Briefly, 5mg DPPH was added into a 25mL volumetric flask, and diluted with DMSO to 25mL. The volumetric flask was covered by a black plastic bag and shaken fully to obtain the DPPH stock solution. Then, taking out 1.8mL DPPH stock solution and diluting with 8.2mL DMSO to 10mL as the working solution, which was measured the absorbance as A_0 at 519nm. Subsequently, 40μL *P. nagi* kernel oil was added into 5mL fresh DPPH stock solution and shaken to mix well, which was also measured the absorbance as A at 519nm. The DPPH scavenging ability was calculated at the ratio [(A_0-A) / A_0 $\times$ 100], each experiment was repeated for 5 times.

2.3.2 Preliminary *in vitro* anticancer evaluation

The anticancer activity of this oil was evaluated against gastric cancer, breast cancer (MCF-7), lung cancer(A549) and Hela cell lines using the counting kit-8 (CCK-8) method [7]. The evaluation process was described elsewhere with some modifications. Briefly, the oil was dissolved in DMSO at a concentration of 100mg/mL, then diluted successively with DMSO for eight different concentrations (100 mg/mL, 50 mg/mL, 25 mg/mL, 12.5 mg/mL, 6.25 mg/mL, 3.125 mg/mL, 1.5625 mg/mL and 0.78125 mg/mL, respectively) as stock solution for below experiment.

The Procedure for Anticancer Evaluation

The target cancer cell lines were seeded in 96-well plates (5000cells/well) with 100μL DMEM supplemented with 10% fetal bovine serum, and cultured at 37 °C in a humidified CO_2 incubator (95% air, 5% CO_2) for 24h. While the cell lines grew to 90% in logarithmic growth, the culture medium was removed from each well, and 100 μL fresh DEME was added to each well. Then, 10μL different concentrations of *P. nagi* kernel oil solutions were added into each well (every concentration was repeated for 5 times) and the plates were incubated for another 48h at 37 °C. Subsequently, 10μL CCK8 was added to each well, and the plates were cultured at 37 °C for another 4 hours. The optical density was measured at a wave-length of 450 nm on an ELISA microplate reader. DMEM and DMSO solution (V/V: 10/1) was used as a negative control. The results were expressed as the inhibition calculated at the ratio [(1-(OD_{450} treated/ OD_{450}negative control)) $\times$100].

3. Results and discussion

3.1 The influence of steaming time on the yield and quality of *P. nagi* kernel oil

The *P. nagi* kernel oil was obtained through two steps: (1) steaming the powder of *P. nagi* kernel; (2) squeezing the steamed powder of *P. nagi* kernel. During the experiment, we found that the time of steaming of the powder of *P. nagi* kernel is extremely important for the yield and the quality of the oil, the short time steaming does not lead to the higher yield, while long time steaming will lead to the higher yield, but the oil also contains higher water content, which affects the quality of the oil, making the oil very turbid. Thus, we optimized the experimental conditions about the steaming time, the yield and the quality of oil. The results (see to**table 3**) exhibited that the yield of oil increased with the increasing of the steaming time, and the steaming time is 20min with the 90% yield of oil, and the oil quality is normal; even if the steaming time

is 25min with the 93% yield, the oil quality is turbid and the yield increased only 3% compared with the 20min steaming time. Thus, we selected steaming time 20min as the optimized condition.

Table 3 . Steaming time of *P. nagi* kernel, yield and oil quality

Steaming time (min)	5 min(±2)	10 min(±2)	15 min(±2)	20 min(±2)	25 min(±2)
yield	60%	65%	75%	90%	93%
quality	clear and transparent	clear and transparent	clear and transparent	clear and transparent	turbid

3.2 The components in *P. nagi* kernel oil

3.2.1 The organic components in*P. nagi* kernel oil

We used the standard methods to analyze the chemical components of this oil. The results listed in **table 4** . We also compared the components of this oil to other edible oils, which can provide the evidence whether the *P. nagi* kernel oil can be used for food. From **table 4** , we can find that the palmitoleic acid content in*P. nagi* kernel oil is much lower than that of camellia oil, olive oil, peanut oil and cottonseed oil; while the content of (9*Z* ,12*Z*)-9,12-octadecadienoic acid in *P. nagi*kernel oil is much higher than that of camellia oil, olive oil and rapeseed oil, but almost the same as that of the peanut oil; this oil also contains higher content of *cis* -11-eicosenoic acid and*cis* -11,14-eicosadienoic acid, while these two ingredients don't contain in other listed oils, even in peanut oil; what's the most important is that the *P. nagi* kernel oil contains higher contents of flavonoids and vitamin E, which are very beneficial to human health.

Table 4 . The components of*P. nagi* kernel oil confirmed in this study and

the comparison of other edible oil

No.	*P. Nagi* kernel oil	*P. Nagi* kernel oil	*P. Nagi* kernel oil	*P. Nagi* kernel oil	The oils used in food field	The oils used in food field	The oils used in food field	The oils used in food field	Th oil use foo fie
	Testing items	Testing items	Unit	Testing result	Camellia oil	Olive oil	Peanut oil	Rapeseed oil	Co oil
1	Total flavonoids (calculated as rutin)	Total flavonoids (calculated as rutin)	%	4.23	–	–	–	–	–
2	Fatty acids	Palmitic acid (C_{16}:0)	%	3.41	8.8	9.5	11.4	2.3	17. 23.
		Palmitoleic acid (C_{16}:1)	%	0.0365	0	0	0	0	0
		Heptadecylic acid (C_{17}:0)	%	0.0520	0	0	0	0	0

No.	*P. Nagi* kernel oil	*P. Nagi* kernel oil	*P. Nagi* kernel oil	*P. Nagi* kernel oil	The oils used in food field	The oils used in food field	The oils used in food field	The oils used in food field	Th oil use foo fie
		Heptadecenoic acid (C_{17}:1)	%	0.0531	0	0	0	0	0
		Stearic acid (C_{18}:0)	%	1.34	0.8-1.1	1.4	3.0	2.3	0.9
		Oleic acid (C_{18}:1)	%	27.6	82-86	81.6	41.2	15.8	22. 44.
		(9*Z*,12*Z*)-9,12-Octadecadienoic acid (C_{18}:2)	%	38.2	7.4	7.0	37.6	14.6	33. 50.
		Arachidic acid (C_{20}:0)	%	0.111	0	0	0.67	0	0
		cis-11-eicosenoic acid (C_{20}:1)	%	1.40	–	–	–	–	–
		(9*Z*,12*Z*,15*Z*)-9,12,15-Octadecatrienoic acid	%	0.215	0.2	0	0	9.2	0
		cis-11,14-eicosadienoic acid (C_{20}:2)	%	8.04	0	0	0	0	0
		ω-3	%	0.2	0.7	–	0	0	–
		ω-6	%	48.0	11.6	4-7	22-28	5-10	–
		ω-9	%	21.0	76.8	84-86	50-68	70-80	–
		Saturated fatty acids	%	4	10.5	9-11	17-18	5-10	25
3	Vitamin E	Vitamin E	mg/100g	2.11	–	–	–	–	–

"—" means that no relevant report data.

3.2.2 Trace elements in *P. nagi* kernel oil

The trace elements in this oil was confirmed using ICP-MS. Firstly, series of standard working solutions were successively measured to build the standard curve, and the regression equations were obtained accordingly. The regression equation, correlation coefficient and linear range of each element are shown in **table 5** .

The trace elements in this oil was tested accordingly and the contents were calculated according to the regression equations and the results also showed in**table 5** . From **table 5** , we can know that*P. Nagi* kernel oil is rich in nine essential microelements beneficial to human health (it is reported that 18 kinds of essential microelements have been conformed as necessary to human health and life, namely, iron, copper, zinc, cobalt, manganese, chromium, selenium, iodine, nickel, fluorine, molybdenum, vanadium, tin, silicon, strontium, boron, rubidium, arsenic, etc.[8]).

Table 5. Regression equation, correlation coefficient (R) and detection limit

and the contents of trace elements in *P. nagi* kernel oil

Element	Internal standard element	Regression equation	R	Detection limit (ng/mL)	Content of each element in oil (μg/g)
V	Sc	Y=1.0592X+0.0440	0.9992	0.07894	0.006
Cr	Sc	Y=1.4904X+0.7925	0.9992	0.3038	0.024
Mn	Sc	Y=0.4714X+0.2261	0.9994	0.3177	40.109
Fe	Sc	Y=1.0349X+46.4863	0.9988	7.313	2.292
Co	Sc	Y=3.0012X+0.1006	0.9989	0.02179	0.007
Zn	Ge	Y=0.2807X+1.6809	0.9986	0.4763	4.361
As	Ge	Y=0.2098X+0.0329	0.9975	0.3918	0.009
Se	Ge	Y=0.0045X+0.0050	0.9927	5.783	0.240
Sr	Rh	Y=0.0120X+0.0023	0.9959	0.1689	0.453

3.3 IR analysis of the obtained oil

The obtained *P. Nagi* kernel oil was also carried out the FT-IR analysis. The FT-IR spectra displayed in **figure 1** . It appeared a weak =C-H stretching band at 3008cm^{-1}, because the oil contains the unsaturated fatty acids and flavonoids, which contain the =C-H groups; the strong stretching bands at 2854-2953cm^{-1} were confirmed as C-H, for there are many saturated C-H bonds in the molecules in this oil; the strong stretching band at 1747cm^{-1} was confirmed as -C=O and -COOH, for the oil contains different kinds of unsaturated fatty acids and saturated fatty acids(which contain COOH group) together with flavonoids(which contain C=O group);the medium stretching band at 1463cm^{-1} was attributed to the absorbance of C=C; the medium stretching bands at 1099-1236cm^{-1} were attributed to the bending vibration of C-H and the medium stretching bands at 723cm^{-1} was the characteristic peak of benzene (which was attributed to the flavonoids). The peak signals of FT-IR are consistent well with the functional groups of compounds listed in **table 4** .

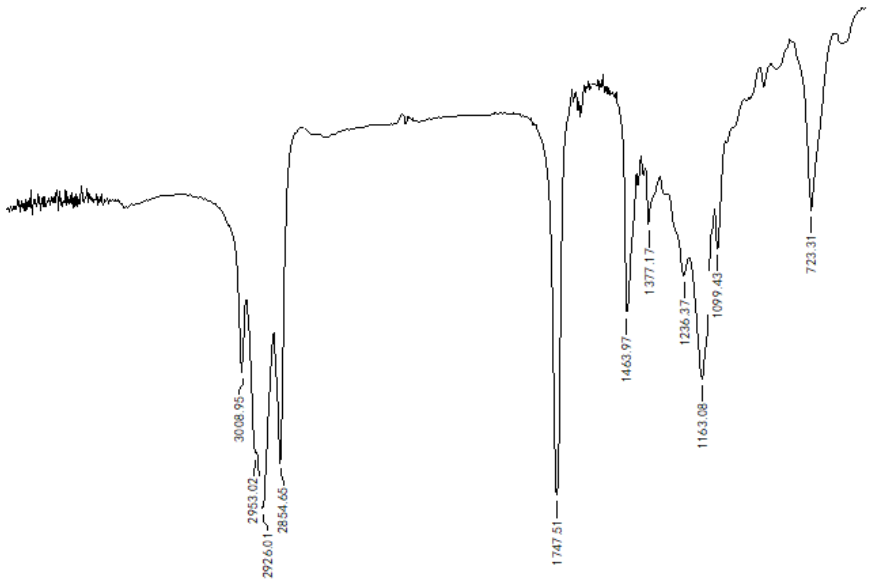

Figure 1 . FT-IR spectra of *P. Nagi* kernel oil in the frequency range 4000-500cm^{-1}

3.4 *In vitro* biological evaluation

3.4.1 Antioxidant effect of*P. nagi* kernel oil

The radical scavenging ability of *P. nagi* kernel oil was evaluated in the conventional system of DPPH[?]. Firstly, we added 40μL*P. nagi* kernel oil to different concentrations of DPPH respectively and studied the scavenging ability to optimize the best experimental conditions. The results (**Figure 2**) showed that the optimized DPPH concentration is 0.036mg/mL and the scavenging ability is 86.56%; Then, we also added different amounts of *P. nagi* kernel oil to 5mL DPPH solution to optimize the optimal concentration of *P. nagi* kernel oil with the highest scavenging ability. The results (**Figure 3**) showed that the *P. nagi* kernel oil exhibited the scavenging ability in a concentration-dependent manner from 5μL to 40μL and exhibited the highest scavenging ability at 40μL with 85.56%. Then, we tested the scavenging ability of *P. nagi* kernel oil at the optimized conditions: 5 mL of 0.036mg/mL DPPH solution, *P. nagi* kernel oil 40μL, the three times of scavenging abilities are: 85.07%, 85.67% and 84.80%; the average scavenging ability is 85.18%.

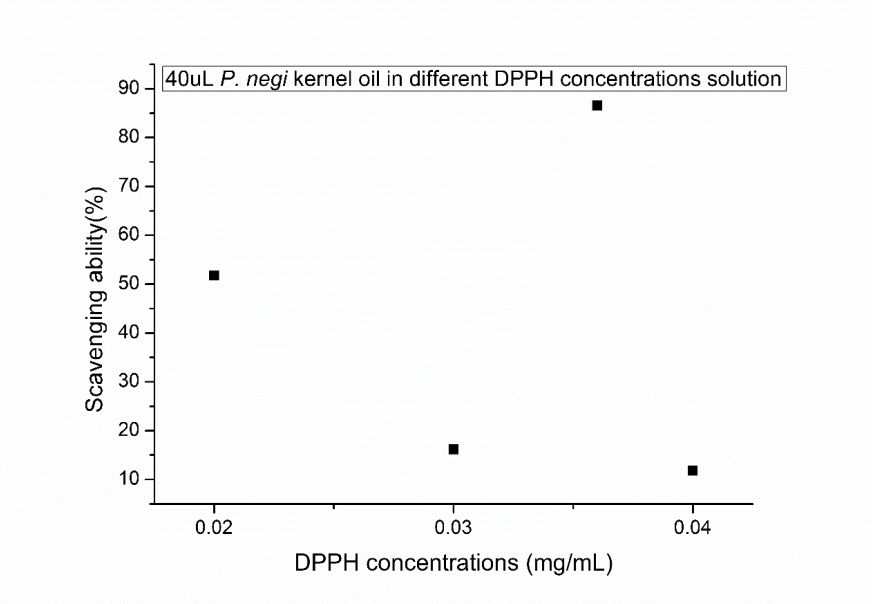

Figure 2 . The scavenging ability of *P. nagi* kernel oil at different DPPH concentrations

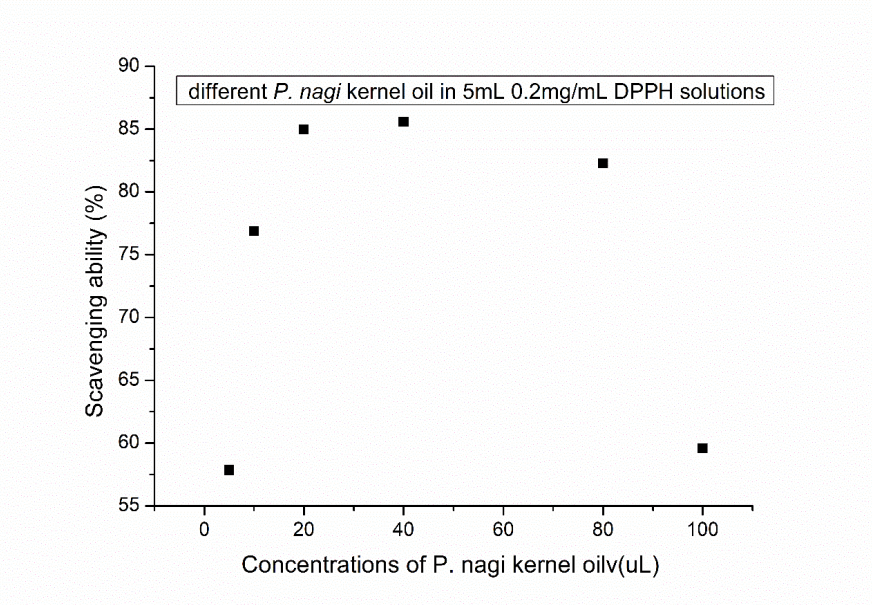

Figure 3 . The curve of concentrations of *P. nagi* kernel and DPPH scavenging ability

3.4.2 Preliminary *in vitro* anticancer evaluation

The preliminary *in vitro* anticancer result of*P. nagi* kernel oil against gastric cancer, breast cancer (MCF-7), lung cancer(A549) and Hela cell lines listed in **table 6** . It showed that *P. nagi* kernel oil exhibited stronger anticancer activity against these four cancer cell lines: the anticancer efficacy against gastric cancer cell lines is in a concentration-dependent manner from 0.78125 to 25mg/mL, and the highest inhibition is 64.30%±2.80 with IC_{50}=48.47mg/mL; the anticancer efficacy against lung cancer(A549)

cell lines is in a concentration-dependent manner from 0.78125 to 50mg/mL, and the highest inhibition is 93.21%±2.24 with IC_{50}=11.47mg/mL; the anticancer efficacy against breast cancer(MCF-7) cell lines is in a concentration-dependent manner from 0.78125 to 25mg/mL, and the highest inhibition is 52.86%±2.57 with IC_{50}=736.14mg/mL; while the anticancer efficacy against Hela cell lines is in a concentration-dependent manner from 0.78125 to 50mg/mL, and the highest inhibition is 85.33%±3.03 with IC_{50}=23.77mg/mL. The results imply that this oil can be also regarded as the anticancer agent.

Table 6 . Preliminary *in vitro* anticancer results

Entry	Concentration(mg/mL)	Inhibition (%)±SD	Inhibition (%)±SD	Inhibition (%)±SD	Inhibition (%)±
		gastric cancer	A549	MCF-7	Hela
1	0.78125	13.57±2.65	12.03±1.13	9.76±2.84	6.34±2.58
2	1.5625	16.38±1.91	15.99±2.77	31.90±4.79	11.73±0.94
3	3.125	26.54±1.48	24.02±2.41	35.54±3.46	12.49±3.12
4	6.25	39.90±2.26	34.98±1.69	35.61±2.55	19.61±2.72
5	12.5	44.64±2.41	41.89±4.76	47.20±4.59	23.62±3.01
6	25	64.30±2.80	51.79±2.48	52.87±2.57	34.66±3.38
7	50	61.32±5.32	93.21±2.24	27.93±3.89	85.33±3.03
8	100	27.38±2.63	78.92±2.53	26.33±4.13	77.54±2.08
IC_{50}(mg/mL)	IC_{50}(mg/mL)	48.47	11.47	763.14	23.77

4. Conclusion

In this work, we optimized the optimal conditions for preparing the*P. nagi* kernel oil and its chemical constituents were also confirmed. By comparing the constituents of this oil to other oils used in the field of food, this oil contains many ingredients and trace elements beneficial to the human health and can be also used in the filed of food. In addition, this oil exhibited higher antioxidant and anticancer activities, which imply that this oil can be regarded as the functional edible oil.

Acknowledgement

This work was financially supported by the National Natural Science Foundation of China (21875252) and Self-created Area Project of Major Science Technology Innovation Platform of Xiamen(3502ZCQ20171002).

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Figure 1.pdf available at https://authorea.com/users/374986/articles/492353-preparation-chemical-constituents-of-podocarpus-nagi-kernel-oil-and-its-in-vitro-antioxidant-and-anticancer-activities

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