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Valorisation of phytochemical from Sitka spruce (*Picea sitchensis*) needles: Impact of ultrasound/ microwave-assisted extraction

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ABSTRACT

Sitka spruce (*Picea sitchensis*) needles contain a variety of bioactive compounds including phenolic compounds and flavonoids, many of which have been used in the cosmetic, pharmaceutical, and food industries. This study aimed to investigate the effects of novel extraction techniques, including ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE) and simultaneous ultrasound–microwave-assisted extraction (UMAE) on the recovery of phenolic, flavonoids and associated antioxidant and anti-cancer properties from Sitka spruce (*Picea sitchensis*) needles. The ferric reducing antioxidant power (FRAP) assay was used to evaluate the antioxidant capacity, and the Alamar Blue assay using the human brain glioblastoma cancer cell line (U-251 MG) was used to evaluate the cytotoxicity activity. Results showed that US-probe accomplished the highest recovery of phenolic and flavonoids at 38 W cm^{-2} for 10 min (106.3 ± 2.5 mg GAE g^{-1} DW and 63.2 ± 3.8 mg QE g^{-1} DW, respectively). Hence, the highest cytotoxicity activity of IC_{50} (0.0114% w/v) was achieved by US-probe at 19 W cm^{-2} for 10 min. However, the antioxidant capacity of (2591.3 ± 92.5 mM TE g^{-1} DW) was achieved under UMAE at ultrasound intensity of 38 W cm^{-2}, microwave power of 302.4 W for 10 min. This study emphasised the potential application of UAE and MAE in the extraction of bioactive as an environmentally friendly method to be used in the valorisation of by-products in food and agro-industries. This supports the use of renewable natural resources in an efficient way to produce high-value compounds therefore it is in line with the new era of bioeconomy and its new biorefinery concepts

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1. Introduction

There are a generation of five billion tonnes of biomass residues annually from food and agroforestry, with 3.3 billion tonnes of CO_2 emissions. EU generate up to 100 million

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tonnes of biowaste per year (Bhardwaj et al., 2021). As per United Nations sustainable development goals by 2030, 4/17 goals are impacted by the agroforestry (Ferreira-Santos et al., 2020). In Europe, the forest is the most abundant biomass feedstock, with approximately 182 million hectares of forest, which generate a turnover of more than 486 billion euros (Trifol et al., 2021). This generates high biowaste yields (needles, cones and bark), which have a negative ecological impact that can be utilised under the biorefinery concept. There is a need for the valorisation of conifer's by-products (e.g., conifer needles and leaves) as a rich and sustainable source of raw materials for phytochemical compounds. Some studies reported the valorisation of bark, needles and cones from various pine species (Kilic et al., 2011; Dubey et al., 2017; García et al., 2018).

Conifers are woody with needle-shaped, single-veined leaves and consist of both male and female unisexual cones. They are comprised of eight families, 70 genera and 630 species (Bhardwaj et al., 2021). Phytochemical components within conifers comprised alkaloids, phenolics and terpenoids (Tanase et al. 2019; Kopaczyk et al. 2020). In Ireland, forestry occupies 770,020 ha of land area as stated by the National Forest Inventory (NFI) (*gov.ie* NFI, 2021). Moreover, coniferous species cover a total of 479,530 ha, with *Picea sitchensis* (Sitka spruce) occupying 52.4 % of the total forest area, being one of the most productive conifers in Ireland (Farrelly et al., 2009; Redmond and JohnJ, 2021). Conifers are mainly planted for timber production with a sales estimation of over €25 million annually.

Conifer extracts rich in phytochemicals with potential health benefits include anticancer (Sahin Yaglioglu and Eser, 2017), antidiabetic (Lee et al., 2016), anticonvulsant (Iravani and Zolfaghari, 2011), antioxidant (Sharma et al. 2016), anti-inflammatory (Azab et al., 2016) and antimicrobial (Ghaffari et al., 2019) properties and prevention of neurodegenerative diseases such as Alzheimer's disease and Parkinson (Bhardwaj et al., 2021). These health-promoting properties could be used as the basis for the production of high-value functional, nutraceutical and pharmaceutical ingredients with significant economic value.

Nevertheless, the recovery of valuable components is a worthy sustainable goal, and it is essential to achieve it with a sustainable approach. To attain this goal, ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) were used as clean and energy-efficient to meet market trends for natural ingredients. UAE's basic principle is based on a phenomenon known as cavitation. The ultrasound wave propagates through the medium via compression and rarefaction, resulting in negative pressure in the liquid. Once the negative pressure exceeds the tensile strength of the liquid, it causes a formation of cavitation bubbles (Wang and Weller, 2006). These bubbles become unstable and undergo implosive collapse; this is referred to as an acoustic cavitation (Soria and Villamiel, 2010; Izadifar et al., 2019).

MAE uses microwave radiation (frequency between 300 MHz and 300 GHz) that interact by heat with solvent and sample matrix using ionic conduction and dipole rotation. Ionic conduction is the migration of ions when electric fields are applied, the solution is heated by the friction created by solution resistance to the flow of ions. Microwave electromagnetics causes disruptions of hydrogen bonds, improves the migration of dissolved ions and helps solvents diffusion into matrices which assist the extraction of compounds of interest (Mahugo Santana et al., 2009; Wijngaard et al., 2012). Heat causes a significant increase in pressure inside the material, improving penetration of the solvent into the material due to an increase in porosity of the biological material (Tatke and Jaiswal, 2011). The phenomena disrupt cellular structural and functional components, facilitating the release of bioactive from the biological material. These effects improve mass transfer, increase efficiency and improve the diffusion mechanism (Vilkhu et al., 2008; Zhu et al., 2022).

The ultrasound/microwave-assisted extraction (UMAE) can result in faster and more efficient extraction of compounds compared to using either method alone. This is because the microwave energy can provide fast and direct heating of the plant material and solvent, while the ultrasound energy can cause mechanical agitation that can help to release the compounds from the plant material. A combination of UAE and MAE, ultrasound/microwave-assisted extraction (UMAE), is a complementary and more effective technique with a higher extraction yield.

The aim of the present study is to explore the feasibility of employing UAE and MAE in bioactive compounds extraction from conifer species harvested from the Irish forest and characterise the bioactivities of the extracts to evaluate the quality of this valorisation process. This support the new era of bioeconomy and its new biorefinery concepts.

2. Results and discussion

2.1. Proximate analysis

The proximate composition of *P. sitchensis* is presented in Table 1 To the best of the author's knowledge this is the first reported value.

2.2. Extraction yield of total phenolic and flavonoids by different extraction methods

The extraction yields of TPC and TFC for UAE, MAE and UMAE are shown in Fig. 1. For UAE, the content of TPC and TFC were from 63.1±2.1–106.3±2.5 mg GAE g^{-1} DW and from 41.9±2.4–63.2±3.8 mg QE g^{-1} DW, respectively. The highest extraction of bioactive was achieved using US-probe at an intensity of 38 W cm^{-2} for 10 min and was significantly higher ($p < 0.05$) by 82 % and 92 % for TPC and TFC, respectively. It was observed that higher ultrasonic intensity and frequency enhanced the phenolic and flavonoid content, which agreed with previously reported results (Tiwari, 2015). This can be attributed to the cavitation phenomena of the ultrasound waves. As the ultrasound amplitude/intensity increases, a violent implosion of cavitation bubbles produces high-velocity interparticle collisions, macroturbulence and perturbations in the microporous particles of the biomass. Hence, this

Table 1 – Proximate composition of *P. sitchensis* plant powder.

Parameters	% dry weight
Protein	21.8 ±0.03
Fat	5.00 ±0.50
Carbohydrates	33.80 ±0.30
Moisture	31.60 ±0.01
Ash	3.00 ±0.001
Others	4.5

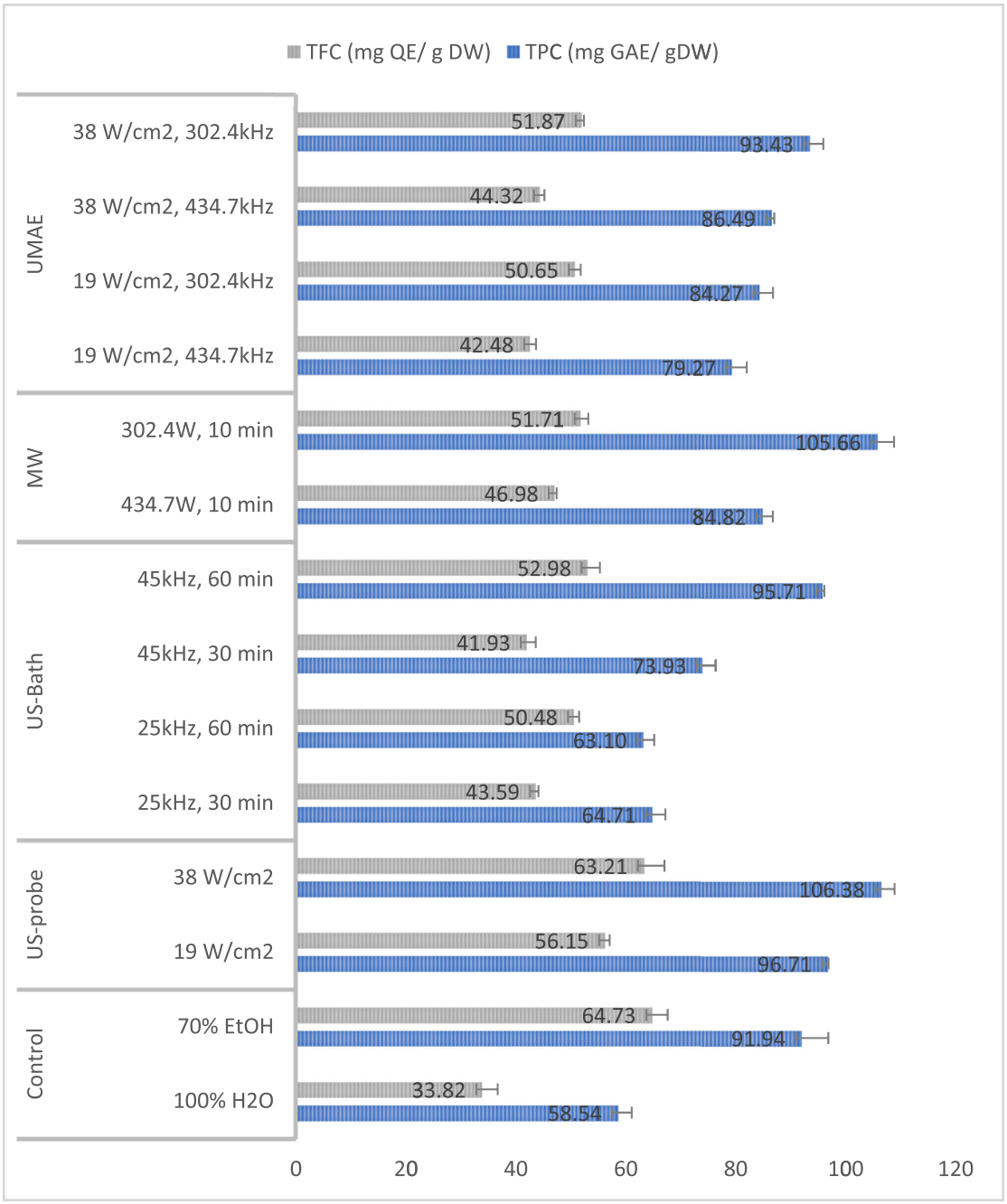


Fig. 1 – The total phenolic (mg GAE/ g DW) and flavonoid (mg QE/g DW) content obtained from the *P. sitchensis* needles supernatant was extracted with different methods. Each value presents the mean value ± SD. The figure was prepared using Microsoft Excel (Version 16.39, Microsoft 365 subscription, data analysis 2019 software).

collision results in erosion and peeling, improving the disruption of the P. sitchensis sample, enhancing the mass transfer, and increasing yield and efficiency. TPC and TFC yields in US-probe increased by 10 % and 12.5 %, respectively, as the power increased from 19 W cm^{-2} to 38 W cm^{-2}.

For US-bath, the extraction time showed no significant ($p > 0.05$) effect on the yield of TPC at 25 kHz ultrasound frequency but had a significant ($p < 0.05$) effect on the TPC at 45 kHz. For TFC, the extraction time affect the yields at both ultrasound frequencies 25 kHz and 45 kHz. There was a slight increase in the yield of TPC (30 %) and TFC (26 %) at 45 kHz ultrasound frequency when extraction time was extended from 30 min to 60 min. However, considering the energy and time, this increase is not efficient.

The results obtained from ultrasonic treatments show that the US-probe has a better impact on the extractability of phenolic and flavonoids than US-bath; this indicates that ultrasonic power plays a significant role in the extraction. The US-bath generates sonication using ultrasonic transducers placed in multiple locations within the tank. Hence, the transducer in the US-bath has a large surface area to vibrate and generate a sound wave in the medium at low intensity (Wen et al., 2020). Compared to the US-probe, the transducer is attached to the probe, which provides a direct sonication once immersed into the medium. This will reduce mass transfer and minimise the energy loss; furthermore, the small surface area of the probe provides higher ultrasonic intensity, which affects the extraction efficiency (Wen et al., 2018).

Previously reported studies have shown the effect of the UAE on the extraction yield of phenolics. Chemelova et al. (2020) reported the optimum phenolic content of extracts by

UAE from Picea abies. The optimal parameters were ultrasound frequency of 35 kHz, 63 °C, methanol content of 53 % (v/v) and 1:38, w/v. The results yielded up to 7.1 times higher phenolic than solvent extraction. Similarly, Spinelli et al. (2019) investigated the effect of UAE and other green technologies for extracting bioactive from Picea abies (Norway spruce) bark. The UAE conditions were US-bath, 39 kHz, 54 °C, 70 % ethanol, and 60 min. Results showed a 64 % increase in TPC and a 49% increase in FRAP compared to various green extraction techniques applied. The extraction of phenolic compounds from maritime pine wood (sawdust waste) increased by 40 % when UAE was applied, compared to solvent extraction, and reduced the time of the process (Meullemiestre et al., 2016).

For MAE, the yield of bioactives was significant ($p < 0.05$) compared to control. The highest TPC and TFC were 105±3.2 mg GAE/g DW and 51.7±1.5 mg QE/g DW, respectively. The highest extraction of TPC and TFC was achieved at a low microwave level of 302.4 W for 10 min. In addition, the effect of microwave power on the yield of phenolics and flavonoids was evaluated. The yield differences between different power levels were significant ($p < 0.05$) with the same extraction time. However, increasing in microwave power showed no significant ($p > 0.05$) increase in the yield of TPC or TFC. A study reported that an increase in MW power will increase the temperature of the system, resulting in a higher extraction yield. An increase in temperature causes a decrease in viscosity and surface tension hence, an increase in solvent power, enhancing the solubility between solvent and solute, and improving matrix penetration (Hamid Nour et al., 2021). Chan et al. (2011) reported that the extraction yield increased with microwave power. However, this increase may cause a degradation in thermal sensible compounds, hence low extraction yield is achieved. Generally, the yield starts to be insignificant or decreases once the yield reached a certain level.

Microwave power is also related to the quantity of sample and the extraction time required. The power provides localised heating in the plant sample which acts as a driving force for MAE to destroy the plant sample and allows for solute /solvent to diffuse and dissolve. Therefore, higher microwave power will generally enhance the extraction yield and result in a shorter extraction time. However, if microwave power is too high, it can result in poor extraction yield leading to the degradation of thermally sensitive compounds in the plant sample (Deo et al., 2015).

Chupin et al., 2015, investigate the use of MAE for the extraction of phenolic, tannins and sugars from maritime pine (*Pinus pinaster*) bark and compared it to hot-water extraction. The results revealed a higher yield and extraction time was significantly reduced from 2 h to 3 min. Moreover, a small particle size of 400 µm improved the extracts obtained. Moreover, MAE was used to extract phenolics from spruce (*Picea abies*) with the effects of particle size (0.3, 1, 2.5 mm), time (3–20 min), and temperature (60, 80, 100°C) on polyphenol recovery were also being investigated. The highest phenolics content is 321 mg GAE 100 g^{-1} with 1 mm particle size and at 100 °C (Sladkova et al., 2016).

Comparing UAE with MAE, the extraction yield of phenolics from both methods was comparable, however, UAE obtained 22% higher flavonoids yield than MAE. Many studies have compared ultrasound and microwave-assisted methods for extraction.

Nisca et al. (2021), reported the optimum phenolics content of extracts by UAE and MAE from *P. abies* bark extracts were significantly different. Results revealed that UAE at 40 kHz ultrasound frequency, 65 °C for time 30 min had higher phenolics and tannins content compared to MAE. However, MAE was found to give the highest content of volatile terpenoids. Liazid et al. (2010), evaluated various extraction techniques for the extraction of bioactive from pine seeds of two species *Pinus maritima* and *Pinus d'Alpes*. The results showed that ultrasound waves, using water as a solvent at 75°C for 20 min, yielded twice the recovery of phenolic compounds compared to MAE.

Simultaneous ultrasonic/microwave-assisted extraction (S-UMAE) was carried out and the extraction yields of TPC and TFC results are shown in Fig. 1. For ultrasound intensities, the results revealed significant ($p < 0.05$) differences in the yield between the two ultrasound intensities under the same microwave power. Increasing the ultrasound intensity from 19 to 38 W cm^{-2} resulted in the increment of the yield within the range between 8.8 % and 10.7 % and 2–4.7 % for TPC and TFC, respectively. For microwave power, the yields for all the groups decreased significantly ($p > 0.05$) with the microwave power increasing from 302.4 to 434.7 W (from 5 % to 7 % and 13–16 % for TPC and TFC respectively). The yield of bioactive was significant ($p < 0.05$) compared to the control. The highest TPC and TFC were 93.4±2.5 mg GAE g^{-1} DW (60 % increase) and 51.8 ±1.2 mg QE g^{-1} DW (54 % increase), respectively. Achieved at 302.4 W microwave power, 38 W cm^{-2} ultrasound intensity and 10 min total extraction time. In conclusion, microwave power and extraction time both generated strong effects on UMAE.

The combination of UAE and MAE is more effective and enhances extraction yield. UAE can improve extraction due to the cavitation phenomena, which facilitate cell disruption and mass transfer, hence enhancing the extraction efficiency. On the other hand, MAE causes direct heat inside the plant system which increases the pressure and temperature and therefore enhance mass transfer. Even though UAE produces mechanical mixing due to cavitation, it also limits its ability to generate high thermal energy, while MAE can increase temperature and deliver rapid heating. Therefore, a combination of the two techniques enhances the extraction process.

Some other research also applied UMAE for extraction and compared it with conventional extraction methods. UMAE was applied for the extraction of bioactives from *Larix decidua* bark and compared to conventional extraction, results revealed that UMAE (optimal conditions: ultrasound amplitude 100 % and microwave power 300 W) increases the extraction yield to double, with a better antioxidant capacity, and reduces the extraction time 47 times (Sillero et al. 2020). UMAE was also applied for the extraction of phenolics from Burdock leaves and compared to maceration, results revealed that UMAE gave the highest phenolics of 9 mg GAE g^{-1} DW where 0.5 mg GAE g^{-1} DW was achieved by maceration(Lou et al., 2011). Similarly, Xu et al. (2018), showed that the extraction yield of pectin from jackfruit peel is 21 % higher by UMAE compared to 17 % yield by conventional extraction method with a reduction in extraction time from 2 h to 30 min by UMAE. In another study, the yield of polysaccharides from *Inonotus obliquus* was extracted with UMAE where a 53 % increase was achieved in comparison to a 14 % increase achieved using a conventional method. The extraction time was reduced from 4 h to 20 min by UMAE (Chen et al., 2010).

The current study, compares the extraction yield of TPC and TFC obtained from UMAE with UAE and MAE alone based on the data discussed. Results showed that combined techniques have a lower extraction yield of TPC and TFC than other methods as in UAE-probe > MW > UMAE. On the contrary, Dong et al. (2021), reported that the highest extraction yield of phenolics from green coffee oils was 16 mg GAE per 100 g sample achieved by MAE followed by UMAE and UAE with an 11 % difference. However, most studies reported that a combination of UMAE can enhance the extraction process by increasing extraction yield and the extract's quality as well as by reducing extraction time, energy and solvent consumed, in comparison to conventional extraction methods or individual extraction methods applied alone. UMAE was applied for the extraction of polysaccharides from *Camptotheca acuminate* fruit. Results showed that a total yield of 6 % was obtained from UMAE, which is 1.75-fold and 1.13-fold higher than UAE and MAE alone, respectively (Sun et al., 2018). Lu et al. (2017) implied the UMAE of oligosaccharides from lotus seeds. Results revealed that the yield of oligosaccharides extracted by UMAE was improved by 76.59 % in comparison to conventional hot water extraction. The extraction time of UMAE was reduced by 1.16, 8.92 and 12.18 times compared to MAE, UAE and conventional extraction.

2.3. *Antioxidant capacity*

In the present study, the antioxidant capacity of P. sitchensis extracts was performed by ferric reducing antioxidant power (FRAP) see Fig. 2. For UAE, the antioxidant capacity was significantly higher ($p < 0.05$) in the US- probe by 27% at 19 W cm^{-2} and 63 % at 38 W cm^{-2} compared to the control sample. For the US-bath, a reduction in the antioxidant power was observed at 25 kHz frequency at both time points

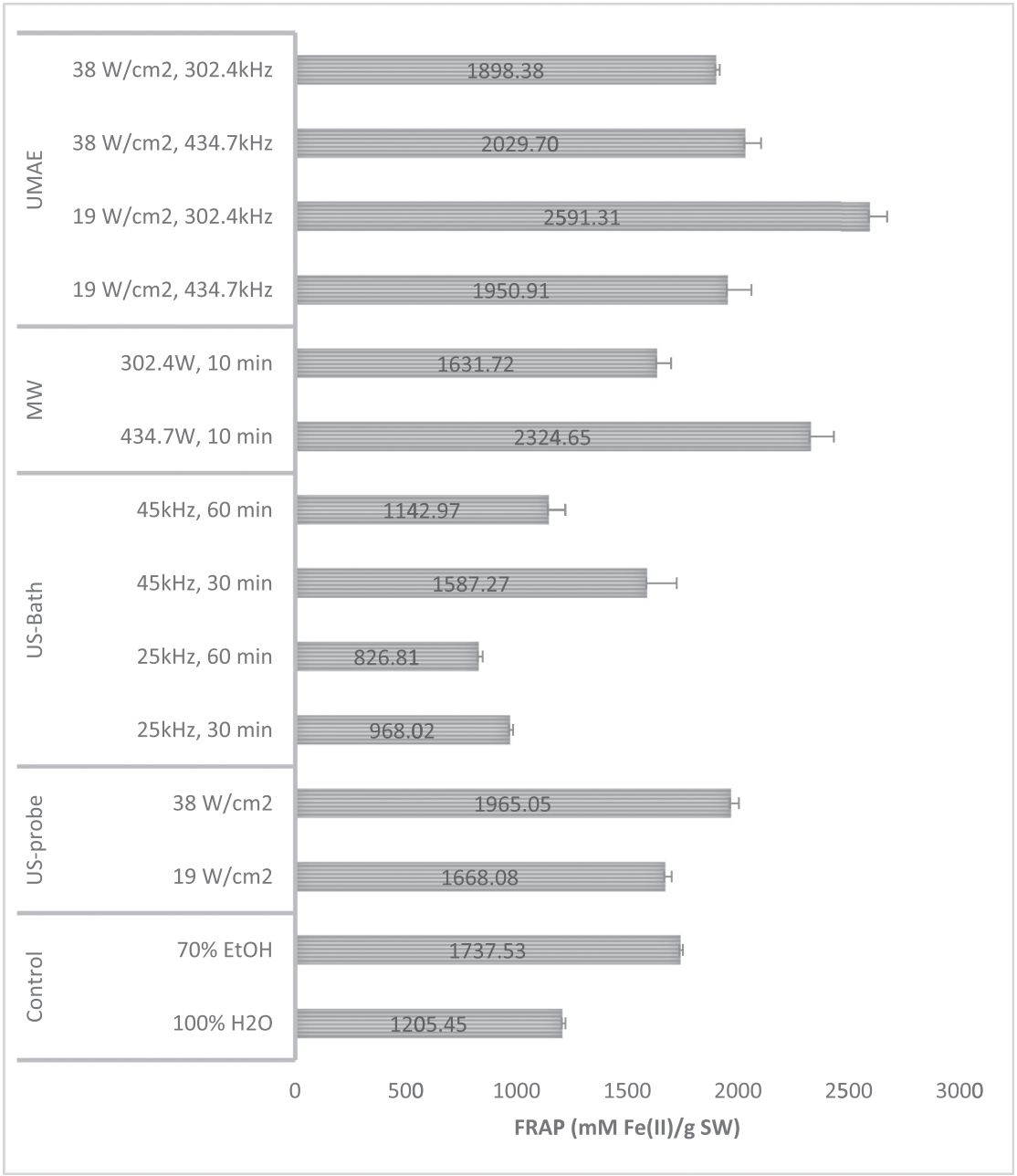


Fig. 2 – Antioxidant activity (FRAP) of *P. sitchensis* needles supernatant extracted with a different extraction method. Each value presents the mean value ± SD. The figure was prepared using Microsoft Excel (Version 16.39, Microsoft 365 subscription, data analysis 2019 software).

Table 2 – Pearson's correlation coefficient of TPC, TFC and FRAP.

Variables	TPC	TFC	FRAP
TPC	1.000	0.758	0.457
TFC		1.000	0.195
FRAP			1.000

and at 45 kHz at 60 min compared to control which can be attributed to sample degradation along with drawbacks associated with the US-bath system. The results above indicate that UAE can enhance the antioxidant capacity, the higher intensity and frequency of UAE in the present study showed benefit to the extraction. In conclusion, according to the results UAE could improve the antioxidant activity of P. sitchensis extracts and shorten the extraction time. Also, it showed that the US-probe at 38 W cm^{-2} gave the highest phenolics and flavonoid content, which positively correlates to the highest antioxidant capacity. The antioxidant capacity of P. sitchensis extracts obtained using deionized water with or without microwave radiation was performed by FRAP assay. The antioxidant capacity was significantly higher ($p < 0.05$) with MW radiation with up to 92% increase with a low MW level of 302.4 W and a 42 % increase with a medium MW level of 434.7 W compared to the control sample. To verify the correlation between TPC, TFC and FRAP, the Pearson correlation analysis was applied and is reported in Table 2. Overall, TPC and TFC showed positive correlation coefficient with FRAP. TPC and TFC in the samples are highly (0.758) correlated to each other, but TPC correlated stronger than TFC with FRAP.

For UMAE, the antioxidant capacity was significantly higher ($p < 0.05$) with up to 114 % increase compared to control. Achieved at 302.4 W microwave power, 38 W cm^{-2} ultrasound intensity and 10 min total extraction time.

2.4. *Cytotoxicity activity*

In the current study, *P. sitchensis* needle extracts that underwent UAE treatment were screened for their cytotoxicity effect against the human brain glioblastoma cancer cell line (U-251 MG) using the Alamar Blue assay over an incubation duration of 24 h, 48 h and 6 days (see Figs. 3–5). The IC_{50} values obtained are summarised in Table 3. The extracts were screened for their cytotoxic effect at different concentrations ranging from 1 % to 0.0004 % (w/v) *P. sitchensis* needle extracts. The negative control, ultrapure water, in which the extracts are dissolved in, and 20% DMSO positive control were used to normalise percentage cell viability. For UAE, preliminary results reveal that *P. sitchensis* needle extracts showed moderate-to-significant cytotoxic activity. The most promising extract required to inhibit 50 % of cell growth is an extract obtained from US-probe. This particular extract presented the lowest IC_{50} value corresponding to extracts with more potency when compared with the other extracts studied amongst the treatment time. US-probe at 50 % amplitude had an IC_{50} value of 0.068 % (w/v) (24 h treatment) and 0.011 % (w/v) (6 days treatment). It can be observed that some of the IC_{50} of 48 h are higher than the 24 h treatment, which could be because the proliferation of some cells continues in the short term when incubated with moderately toxic concentrations. This would lead to a perceived increase in viability as these cells divide, but they also can succumb to slower cytotoxicity kinetic. This pattern was observed with other cytotoxic agents; therefore, 6 days tend to be selected as the endpoint with the cell line.

The hill slope also agrees with this; as shown in Fig. 3. The cytotoxicity data for 48 h and 6 days show a substantially steeper hill slope than those for 24 h. indicating that lower concentrations result in a population of survivor cells that continue to proliferate at this early time point. It's interesting to note that the IC_{50} value significantly decreased (by more than double) during the course of 6 days. Pine needle samples demonstrated more significant cytotoxicity over a shorter period and at 0.1 % (w/v) concentrations. These results can be attributed to a higher susceptibility of the cancer cells studied to one of the anticancer bioactive presents at higher concentrations in the P. sitchensis extract. This showed that P. sitchensis extracts inhibited the proliferation of glioblastoma cells in a time-and dose-dependent manner.

Studies reported in-vitro cytotoxic and antiproliferative potential of extracts from conifer species on human tumour cell lines(Huang et al., 2005; Wu et al., 2011; Hoai et al., 2015). Extracts obtained from Picea abies and Fagus sylvatica were evaluated for their cell viability against A375 melanoma and A549 lung carcinoma cell lines. Results showed a decrease in cell viability for A375 melanoma was achieved at a higher

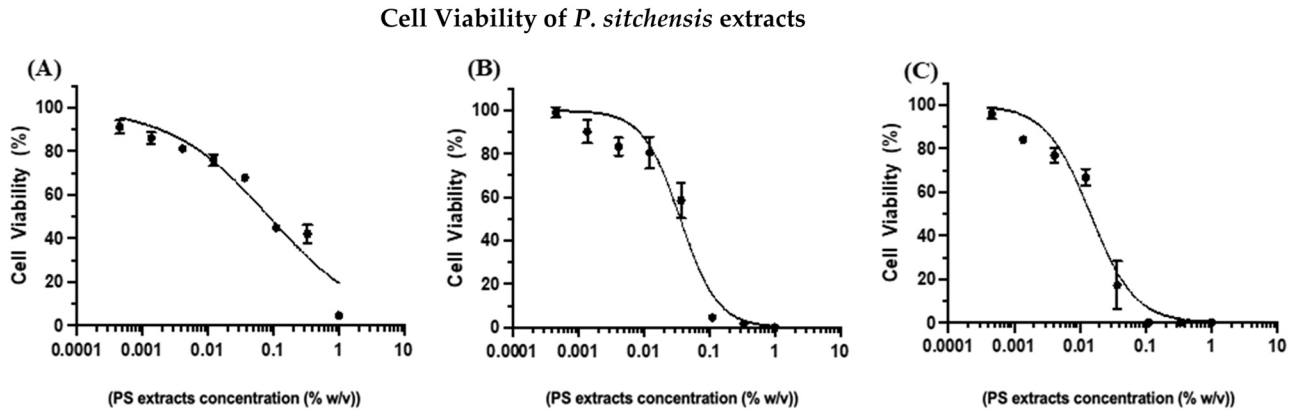
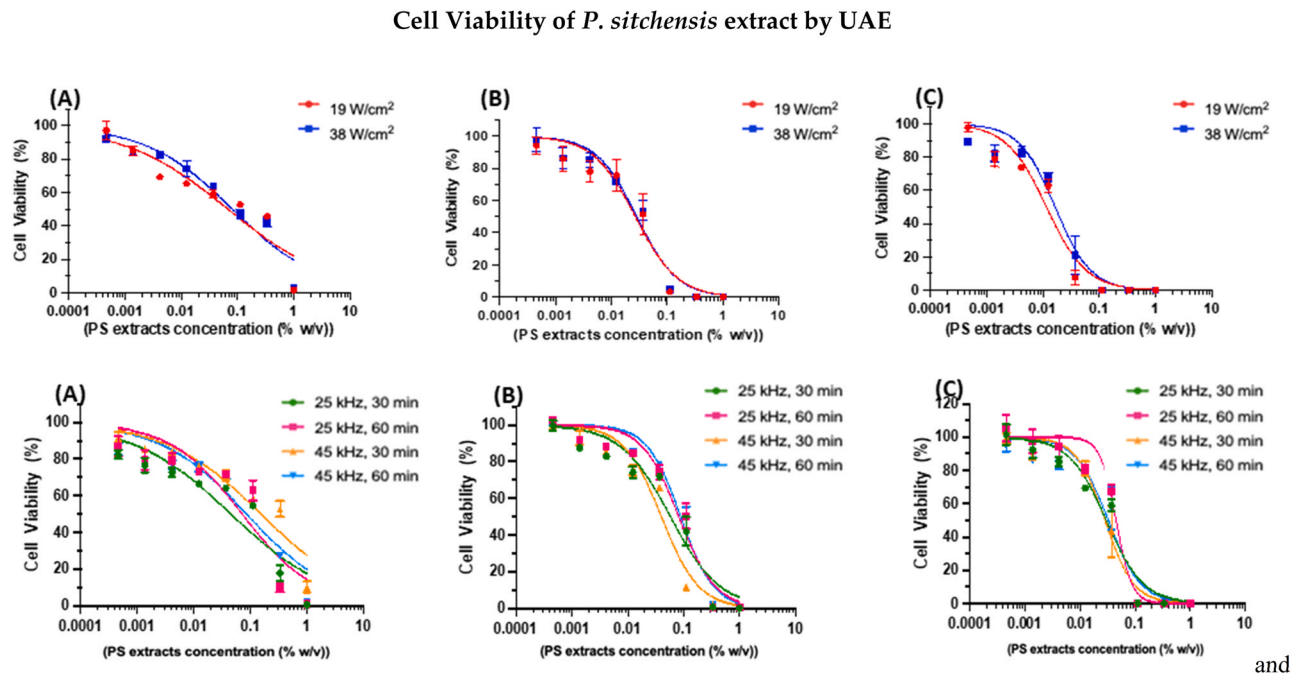


Fig. 3 – (A) 24 h (B) 48 h and (C) 6 days treatment of *P. sitchensis* control extract in U-251MG cells. Cell viability is plotted against the Log10 exponent concentration (% w/v). Data shown was normalised to the untreated control and are shown as mean ± S.E.M. Statistical analysis was carried out using non-linear regression analysis and Two-way ANOVA (*P < 0.05) (n = 3).



and

Fig. 4 – (A) 24 h (B) 48 h and (C) 6 days treatment of the UAE extracts (Top – US-probe; bottom- US-bath) in U-251MG cells. Cell viability is plotted against the Log10 exponent concentration (% w/v). Data shown was normalised to the untreated control and are shown as mean ± S.E.M. Statistical analysis was carried out using non-linear regression analysis and Two-way ANOVA (*P < 0.05) (n = 3).

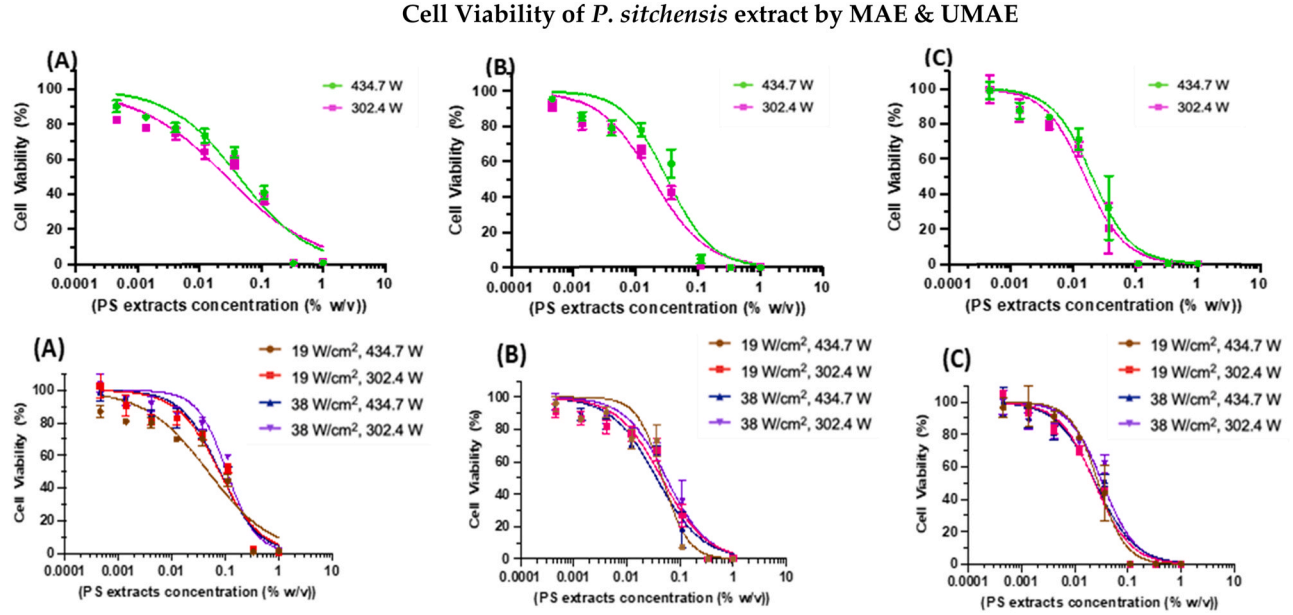


Fig. 5 – (A) 24 h, (B) 48 h, and (C) 6 days treatment of the MAE extracts (Top) and UMAE extracts (bottom) in U-251MG cells. Cell viability is plotted against the Log_{10} exponent concentration (% w/v). Data shown were normalised to the untreated control and are shown as mean ± S.E.M. Statistical analysis was carried out using non-linear regression analysis (n = 3).

concentration (2.5 mg ml^{-1}). No cytotoxic effect was observed on A549 lung carcinoma cells; however, an increase in concentration might improve the activity because it is concentration dependent (Coșarcă et al., 2018). In another study, a range of C-methylated flavone glycoside compounds isolated from *Picea neoveitchii* was evaluated for their antifungal and cytotoxicity activity. The cytotoxicity of all compounds against Spodoptera litura Fabricius cell (SL) was evaluated by MTT assay 4 out of the 9 compounds exhibited potent cytotoxicity inhibition rates between 62 % and 65 % at a concentration of 20 mg $L^{-1.}$ The other compounds showed lower cytotoxicity compared to the control(Chen et al., 2012).

3. Materials and methods

3.1. *Chemicals and reagents*

HPLC grade (> 99.9 %) methanol and chloroform were purchased from Lennox (Dublin, Ireland). Quercetin dihydrate, sodium carbonate, sodium nitrate, hydrochloric acid, gallic acid, sodium chloride, iron (III) chloride hexahydrate, Folin-

Table 3 – Summary of IC_{50} in the percentage of treatment % (w/v; g/ml) extracted from *P. sitchensis* rich extract obtained at 24 h, 48 h, and 6 days treatment in U-251MG cells. IC_{50} ranges show difference of each extract.

Treatment		24 h		48 h		6 days	
		IC_{50} (%)	IC_{50} range (%)	IC_{50} (%)	IC_{50} range (%)	IC_{50} (%)	IC_{50} range (%)
Control		0.08662	0.08118–0.09547	0.03613	0.02025–0.04524	0.01448	0.01139–0.01721
US-probe	19 W cm^{-2}, 10 min	0.06782	0.06127–0.07152	0.02698	0.01150.04088	0.01149	0.01012–0.01329
	38 W cm^{-2}, 10 min	0.08126	0.07573–0.08913	0.02855	0.0216–0.040089	0.01637	0.01442–0.01835
US-bath	25 KHz, 30 min	0.04537	0.04188–0.04887	0.0581	0.04491–0.6681	0.02983	0.0269–0.03326
	25 KHz, 60 min	0.07167	0.04025–0.09218	0.08254	0.07068–0.09572	0.04488	0.04074–0.04788
	45 KHz, 30 min	0.1539	0.1416–0.1688	0.04007	0.03626–0.04569	0.02834	0.01797–0.03944
	45 KHz, 60 min	0.08572	0.06454–0.09822	0.08872	0.0754–0.1156	0.03243	0.024–0.04799
MAE	434.7 W for 10 min	0.04283	0.02976–0.05328	0.03199	0.02301–0.05131	0.01996	0.01316–0.03629
	302.4 W for 10 min	0.02679	0.01766–0.03606	0.01867	0.01494–0.02631	0.01525	0.01305–0.01812
UMAE	19 W cm^{-2}, 434.7 W, 10 min	0.04799	0.03884–0.07371	0.0472	0.02507–0.06564	0.02815	0.01884–0.04664
	19 W cm^{-2},302.4 W, 10 min	0.07722	0.05551–0.1002	0.04544	0.029–0.08037	0.02405	0.02225–0.02729
	38 W cm^{-2},434.7 W, 10 min	0.07933	0.05645–0.09993	0.03672	0.0246–0.05676	0.02492	0.02074–0.02537
	38 W cm^{-2},302.4 W, 10 min	0.107	0.08648–0.01239	0.05564	0.03866–0.07904	0.03376	0.03093–0.03784

Ciocalteau reagent, sulphuric acid, D-glucose, potassium sodium titrate, copper sulphate, Bovine serum albumin (BSA), glacial acetic acid, sodium acetate trihydrate, 2,4,6-tripyridyl-s-triazine (TPTZ), iron sulphate, sodium hydroxide, aluminium chloride, trichloroacetic acid, Dulbecco's Modified Eagle Medium (DMEM), Foetal bovine serum (FBS), trypsin and Ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich (Arklow, Ireland), penicillin-streptomycin solution and phosphate-buffered saline from Merck (Germany).

3.2. *Preparation of plant material*

The needles samples of *P. sitchensis* were collected (Oct- Nov 2021) from Carrigkerry, county Limerick (Ireland). The plant materials were taxonomically authenticated by Professor Trevor Hodkinson in the Botany Department of Trinity College Dublin, the University of Dublin. Needles were deposited from three mature trees and combined. Samples were stored at – 20℃ overnight and subsequently freeze-dried (Thermo Scientific Heto Power Dry LL3000, United Kingdom) at – 50℃ temperature for two days. Samples were then ground to a fine powder (~0.4–0.8 mm) using a mill (MM 400 Retsch, England) and stored at – 80℃ for further analysis.

3.3. *Proximate analysis*

Proximate analysis s carried out according to the following procedures: protein content was determined calorimetrically using Lowry et al., 1951, total fat content was determined gravimetrically using the method described by Folch et al., 1957, total carbohydrate was determined calorimetrically using the method described by Dubois et al., 1956 and ash content (Muffle furnace, ISO 735).

3.4. *UAE, MAE and simultaneous UMAE*

Powdered *P. sitchensis* needles were mixed with deionised water. For UAE, both the US-probe and US-bath systems were employed. An ultrasound probe system (500 W, UIP500hdT, Hislscher, Germany) at 20 kHz with an 18 mm diameter probe was employed. The probe was submerged 30 mm into the sample containing powdered *P. sitchensis* and water. After 10 min ultrasound treatment at 50 % and 100 % amplitudes which correspond to ultrasound intensity of 19 W cm^{-2} and 38 W cm^{-2} respectively, at a ratio of 1:20 (w/v). US-bath system employed for extraction consisted of a stainless steel ultrasonic bath (dimensions: 30 cm length, 24 cm width, 13.5 cm depth, 8.6 L of capacity, operating at two selectable frequencies of 25 and 45 kHz (TI-H-10, Elma, Germany). Treatments were carried out for 30 min and 60 min treatment at a ratio of 1:10 (w/v). MAE was carried out in a modified microwave oven (NN-CF778BPQ, Panasonic) equipped with a condenser and a refrigerated circulator (Grant LTD6/20, Grant Instruments Ltd., Cambridge, UK). The mixture at the ratio of 1:20 (w/v) was prepared in a sealed beaker connected with a condenser. The mixture was irradiated for 10 min at the "low" and "medium" microwave level, 302.4 W and 434.7 W, respectively. UMAE was carried out with the microwave and ultrasound probe mentioned before. Two microwave levels (low and medium) and two ultrasound amplitudes (50%, 100%) were combined at a ratio of 1:20 (w/v) for 10 min. The samples were transferred for centrifugation (10 000g for 20 min). Supernatant after the centrifugation was stored at – 80℃ for further analysis. The Control group had no ultrasound or microwave treatment. All methods employed in the current study were modified from previous study carried out by our colleagues (Wen et al., 2020).

3.5. *Determination of total phenolics and flavonoids content*

The total phenolic content (TPC) of needles extracts was estimated by Folin-Ciocalteu method (Tandon et al., 2011) using gallic acid (0–500 mg L^{-1}) as a standard. Briefly, 50 µl of a sample/standard were mixed with 3.5 ml water, followed by 250 µl 2 N Folin-Ciocalteu reagent. Then, 750 µl of 20 % sodium carbonate solution was added, and the mixture was incubated for 2 h at room temperature. After incubation, the absorbance was measured at 765 nm The total phenolic content was expressed as mg gallic acid equivalents/gram of dry weight (mg GAE g^{-1} DW) (Tandon et al., 2011).

The total flavonoid content (TFC) of needles extracts was estimated by a quercetin dihydrate method(Ghasemzadeh et al., 2012) using Quercetin (0–700 mg L^{-1}) as a standard. Briefly, 100 µl of a sample/standard, 400 µl water, followed by 30 µl of 5 % sodium nitrate. After 5 min, 30 µl of 10 % aluminium chloride was added and vortexed. 200 µl of 1 M sodium

hydroxide and then 240 µl of deionised water were then added. The absorbance was measured at 415 nm. The total flavonoid content was expressed as mg quercetin equivalents per gram of dry weight (mg QE g^{-1} DW).

3.6. Antioxidant activity assay

The ferric-reducing antioxidant power (FRAP) assay is based on the reduction of ferric ion to ferrous ion, which produces a coloured complex ferrous-tripyridyltriazine. The antioxidant power was estimated by FRAP assay(Ghasemzadeh et al., 2012), using Iron (II) sulphate $FeSO_4$ (0–2000 μM) as a standard. Briefly, 200 µl of a sample/standard/blank was added to 3 ml of the FRAP reagent prepared in a 10:1:1 ratio of 300 mM acetate buffer at pH 3.6, 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) and 20 mM ferric chloride ($FeCl_3 0.6\,H_2O$). and incubated for 30 min in the dark. The absorbance was measured at 593 nm using a Biomek Synergy 4 plate reader and the assay was monitored using the Gen5 software. The antioxidant power was expressed in mM Fe(II) equivalent per gram of dry weight (mM Fe(II) g^{-1} DW).

3.7. Cell culture

The human brain glioblastoma cancer cell line (U-251 MG) (ECACC 09063001) cells were obtained from the culture collection of Prof. Michael Carty (Trinity College, Dublin, Ireland). Cells were cultured in Dulbecco's Modified Eagle's Medium-high glucose (DMEM) (Merck, Germany) supplemented with 10% foetal bovine serum (Merck, Germany) and 1% penicillin-streptomycin (Merck, Germany) solution in TC flask T75, standard for adherent cells (Sarstedt, Ireland). The cultures were maintained in a humidified incubator containing 5 % CO_2 at 37 °C. The culture medium was changed every 2–3 days until 80 % confluence was reached. Cells were routinely sub-cultured using a 0.25 % trypsin-EDTA (Merck, Germany) solution. Cells were seeded at a density of 1×10^4 cells/well (24- and 48-hours treatment) or 2.5×10^3 cells/well (6 days treatment) (100 µl culture medium per well, in triplicate in 96-well plates (Sarstedt, Ireland). Plates were incubated overnight at 37 °C with 5 % CO_2 to allow proper adherence. Existing media was removed from each well and cells were then treated with 1% (w/v) stock solution in ultrapure water of each of the different needles extract and serially diluted from 1 % to 0.0004% (w/v), 20 % (v/v) dimethyl sulfoxide (DMSO) was used as a positive control and ultrapure water was used as a negative control.

3.8. Cell viability assay

The Alamar Blue assay encompasses a fluorometric/colorimetric growth indicator based on the detection of metabolic activity. The system incorporates an oxidation-reduction indicator that both fluoresces and changes colour in response to chemical oxidation of growth medium because of cell death. A decrease in cell viability results in a colour change from pink (reduced, fluorescent) to blue (oxidised, non-fluorescent). The Alamar Blue assay (Invitrogen, Ireland) was used to measure the effects of needles extracted against U-251MG cells. After 24 h/48 h/6 days of incubation at 37 °C in 5 % CO_2, the cells were rinsed once with PBS solution and then incubated for 2.5 h at 37 °C with 10 % Alamar blue and 90 % DMEM-high glucose solution. The cell viability was measured by fluorescence (excitation, 530 nm: emission, 590 nm) using a Varioskan™ LUX multimode microplate reader (Thermo Scientific, USA)(Cabral et al., 2021).

3.9. Statistical analysis

Each bioassay experiment was carried out in triplicate and the data were expressed as means ± standard deviation (SD). Statistical analyses were performed on IBM SPSS statistical software version 28. The analysis of variance (ANOVA) with p-values < 0.05 were considered to be statistically significant. Pearson correlation analysis was performed to explore the correlation between TPC, TFC and FRAP colour using XLSTAT (version 2020.3, Redmond, Washington, USA). For cytotoxicity, all assays were performed in triplicate, independently of each other with a minimum of three replicates per experiment. Data shown are pooled and presented as mean ± SEM (n = total number of replicates) unless stated otherwise. Curve fitting and statistical analysis were performed using Prism 9, GraphPad Software, Inc. (USA). Unless otherwise indicated, significant differences were considered with a p-value < 0.05.

4. Conclusions

In the present investigation, several bioassays were performed on the needles of *P. sitchensis* to understand the impact of green technologies on the extraction of bioactive for industrial applications. Conifer species produce a range of secondary metabolites with antioxidants, cytotoxicity and other pharmacological activities. The modes of assays used in this study were both qualitative and quantitative. The cavitation technology improved the extraction efficiency due to the increased mass transfer. Among the extraction techniques studied, UAE at an intensity of 38 W cm^{-2} for 10 min was found to generate the highest yield of phenolic, flavonoid and best cytotoxic activity. Assisted with microwave penetration, more antioxidant compounds were released. Extracts obtained from UMAE at ultrasound intensity of 38 W cm^{-2} microwave power of 302.4 W for 10 min generated the highest antioxidant capacity. More parameters are associated with each extraction technique that requires further investigation and extraction optimisation is necessary.

Most of the studies currently on UAE or MAE are based on lab scales. Since it showed better TPC and TFC extraction yield and quality, in further study, it should aim to ensure that the upscaled conditions would have the optimum input of power and energy in the microwave and the ultrasonic system, by tuning the nominal power or percentage amplitude and the treatment time. It should be noted that these energy consumption parameters are not related to the activation energy of the extraction process. Instead, activation energy that matters during extraction processing refers to a specific portion of the total energy that is delivered to the extraction mixtures to increase the internal pressure of the plant sample during microwave heating or to impact the external plant structure during ultrasonic cavitation to disrupt the plant sample. Nevertheless, energy-based parameters are reliable calibration parameters to indicate the input power and energy required to be set in the microwave and ultrasonic systems to complete an extraction.

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CRediT authorship contribution statement

HA, performed the research work, collected and analysed the data, and prepared the manuscript. TB, PD, BK and MM supervised the research work. XZ, methodology and formal analysis under the supervision of BKT. JRMM, methodology and formal analysis under the supervision of JFC. All authors edited and reviewed the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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