Evaluation of antioxidant and antimicrobial properties of nutshells of walnuts and chestnuts

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Abstract

Recently, there has been a growing interest in the research of the benefits provided by plants like medicinal herbs, becoming a basis for modern medicine (Evans et al., 2002). In particular, there has been an increased focus on the properties of nuts. Aside from containing various nutrients like magnesium and copper, as well as helping to improve cholesterol, they are also rich in various antioxidants. However, the shells of these nuts are usually thrown away as waste, especially when being exported to other countries, as they contribute to the weight of the ship. In the case of cashew nut shells, only a small amount of them are used as fuel, while the majority of cashew nut shells are removed during processing and therefore wasted (Prakash et al., 2018). Even so, these by-products from the nuts are still valuable as they hold antioxidants just like the nut kernels, as well as antimicrobial properties.

Antioxidants are able to donate an electron to stabilise free radicals within the body. As an overabundance of free radicals leads to protein damage, membrane lipid and nucleic acid, causing cell apoptosis; antioxidants therefore decrease the likelihood of people developing these conditions by maintaining the balance of free radicals within the body (Hindarto et al., 2017). According to Prakash et al. (2018), analysis of total phenolic content within methanolic extracts from powdered nut by-products revealed that cashew nut and coconut shells, as well as groundnut hulls in particular contain various types of polyphenols that have antioxidant properties, with the methanolic extract of the cashew nut shell containing the most antioxidants.

Besides that, nutshells also contain compounds that behave as antimicrobial agents, which are able to kill or inhibit the growth and spread of pathogens such as bacteria and fungi. According to Flores-Estrada et al. (2019), the disk diffusion and macrodilution

tests using extracts from pecan nuts showed antimicrobial activity against Staphylococcus aureus and Escherichia coli. Furthermore, there was a greater phenolic compound content and antioxidant capacity from the pecan nutshell than in the husk. The methanolic extracts from nut shells mentioned earlier also serve as antimicrobials. (Prakash et al., 2018).

Possible uses of these properties besides direct consumption is the processing of the usually discarded nutshells into a natural means to prevent food spoilage via chemical oxidation and microbial development (Shahidi & Shong, 2010), and a substitute to current synthetic food preservatives, which are detrimental to the human body (Prakash et al., 2018). The fact that the nut shells are usually discarded means that they contribute to agro-solid waste, waste produced from agricultural activities, thus finding more applications for nut shells helps to reduce agro-solid waste as well (Hindarto et al., 2017).

Objective

This study will investigate which nutshells are the most effective in terms of neutralising free radicals, as well as inhibiting bacterial growth, by comparing chestnut and walnut shells.

Hypothesis

The hypothesis of this experiment was that the nutshells of walnuts and chestnuts exhibit antioxidant and antimicrobial properties, with walnut shells having higher antioxidant and antimicrobial properties.

Methods

Preparation of nutshell Extracts

Nuts were shelled using nutcrackers, and the shells were grinded into powder by a blender. 1g of nutshell powder were dissolved in 1ml of methanol, ethanol and hexane. This preliminary concentration yielded minimal crude extract as seen in Fig 1. As such the concentration was increased and 10g and 20g samples of nutshell powder were

dissolved in 100ml of ethanol and methanol and left overnight. The samples were filtered and the filtrate was left to dry to obtain the extracts.

Growth of bacterial cultures

Bacteria colonies of Staphylococcus epidermidis ATCC 12228 and Escherichia coli ATCC 25922 were inoculated into 10ml of sterile LB (Luria-Bertani) broth in 10ml falcon tubes along with a blank control in a shaking incubator overnight.

Preparation of agar plates

9.5g of Mueller Hinton Agar Powder was dissolved in 250ml of deionised water, stored in the fridge overnight, autoclaved and poured into 17 petri dishes the next day. Inoculated LB broth was then spread over the petri dish agar, and 4 wells were cut out in the agar using a pipette head.

DPPH Assay of Nutshell Extracts

Respective solvents were added again to the extract powder in concentration 500μM, and 2ml of this solution was further dissolved in 4ml of DPPH solution. The solution was placed into a UV-Vis Spectrophotometer at 515nm and the absorbance at 3 minutes was measured.

The formula used to get DPPH scavenging percentage is ((Absorbance of control - Absorbance of sample)/Absorbance of sample)*100%. Methanol was taken as the blank.

For the control antioxidant, 2ml of gallic acid solutions of varying concentrations (100μM, 50μM, 25μM, 12.5μM, 6.25μM) dissolved in 4ml DPPH solution were used to plot the calibration curve.

Antimicrobial test using well diffusion method

100μl of the different solvent extracts were loaded into the wells. The positive control was bleach and the negative control was water. The plates were incubated at 37 degrees Celsius overnight. The zones of inhibition were measured in mm.

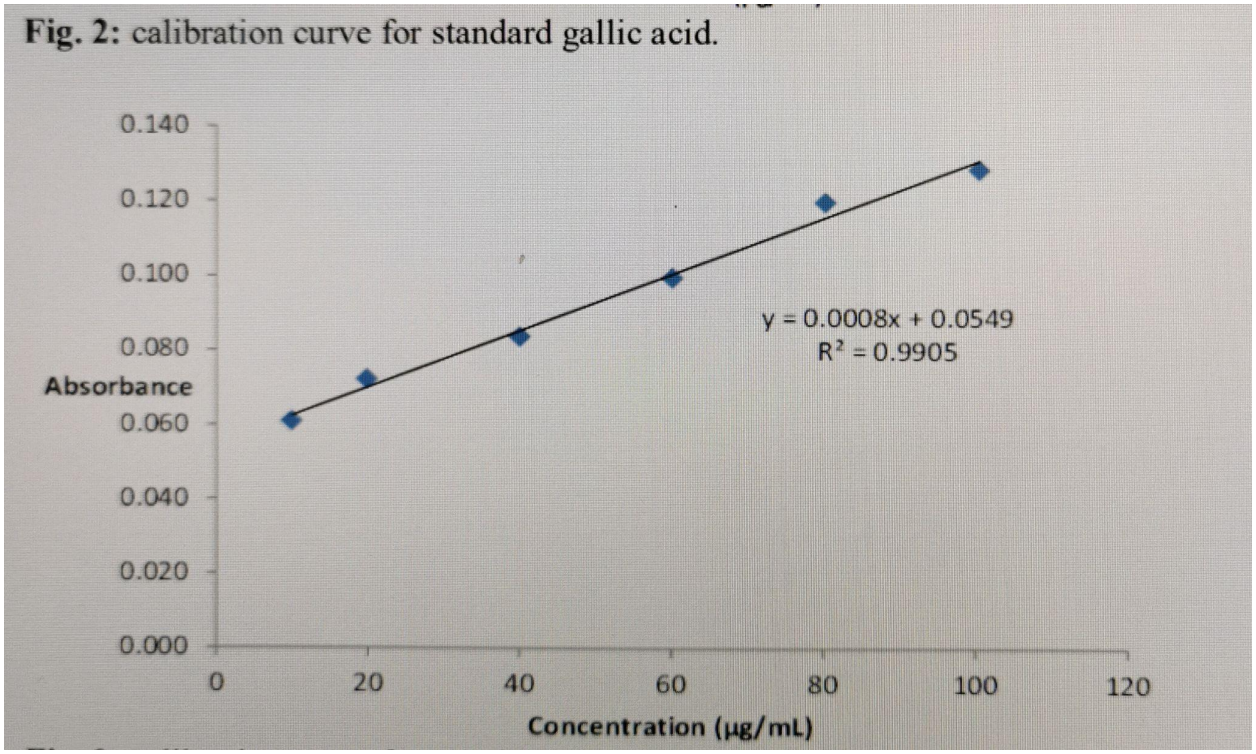

Fig 1: Calibration curve for standard gallic acid. y=0.0008x + 0.0549. R^2=0.9905 taken from (Patricia et al., 2015)

Results analysis

Results of DPPH Assay

Sample	Absorbance after 3min	DPPH scavenged (%)	Antioxidant activity (Gallic Acid Equivalent)(µg/ml)
walnut methanol	0.072	((0.269 - 0.072)/0.269)*100=73.234%	16µg/ml±1.0µg/ml
walnut ethanol	0.098	((0.269 - 0.098)/0.269)*100=63.569%	56µg/ml±2.0µg/ml
chestnut methanol	0.15	((0.269 - 0.15)/0.269)*100=44.238%	110µg/ml±10.0µg/ml

chestnut ethanol	0.07	((0.269 - 0.098)/0.07)*100=73.978%	19µg/ml±1.0µg/ml

Table 1: Table showing absorbance readings at 515nm after 3 minutes, DPPH scavenging activity (%) as well as antioxidant activity in GAE (Gallic Acid Equivalent) of each extract.

As seen in Table 1, methanolic walnut extract had a DPPH scavenging activity of 73.234%, ethanolic walnut extract had a DPPH scavenging activity of 63.569%, methanolic chestnut extract had the lowest DPPH scavenging activity of 44.238%, and ethanolic chestnut extract had the highest DPPH scavenging activity of 73.978%. More antioxidants appear to have been extracted by methanol in walnuts, and more antioxidants were extracted by ethanol in chestnuts. Our 2 attempts at plotting the gallic acid calibration curve failed with the R^2 value of the equation less than 0.900. Hence, the results were compared to the calibration curve present in (Patricia et al., 2015) When comparing these results to the gallic acid calibration curve, methanol and ethanolic extracts of walnuts showed an antioxidant activity of 16µg/ml±1.0 GAE µg/ml and 56µg/ml±2.0 GAE µg/ml respectively while methanolic and ethanolic extracts of chestnut showed an antioxidant activity of 110µg/ml±10.0 GAE µg/ml and 19µg/ml±1.0 GAE µg/ml respectively.

Results of Antimicrobial Test

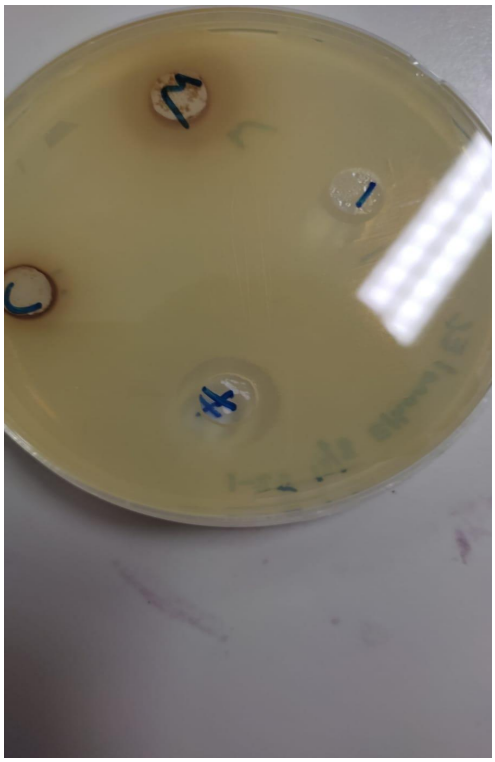

Fig 2: Agar plate shown after conducting well diffusion test of E.coli. W represents the walnut extracted using ethanol. C represents the chestnut extracted using ethanol. + represents 10% bleach. - represents sterile water.

(note that the agar plates as shown are the best results in the triplicates we did)

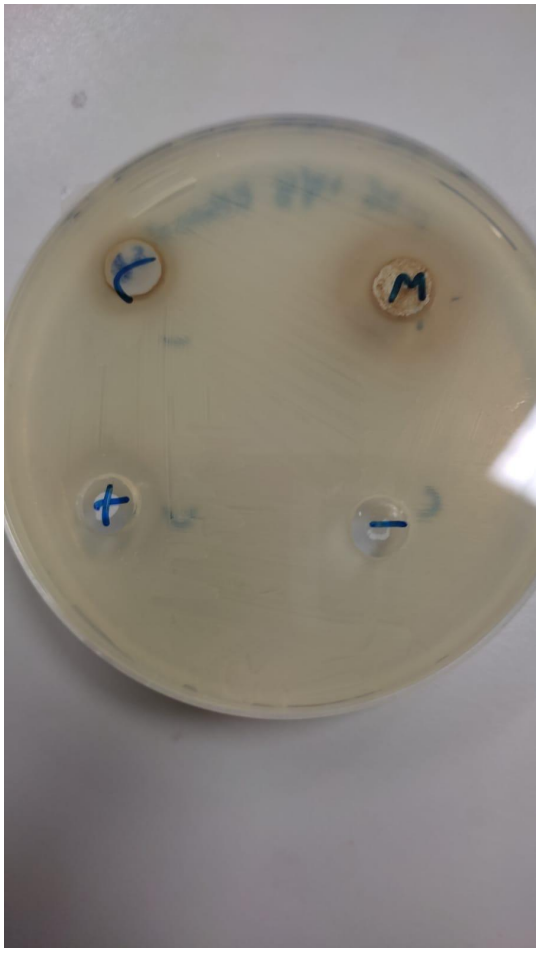

Fig 3: Agar plate shown after conducting well diffusion test of Staphylococcus epidermidis. W represents the walnut extracted using ethanol. C represents the chestnut extracted using ethanol. + represents 10% bleach. - represents sterile water.

(note that the agar plate as shown are the best results in the triplicates we did)

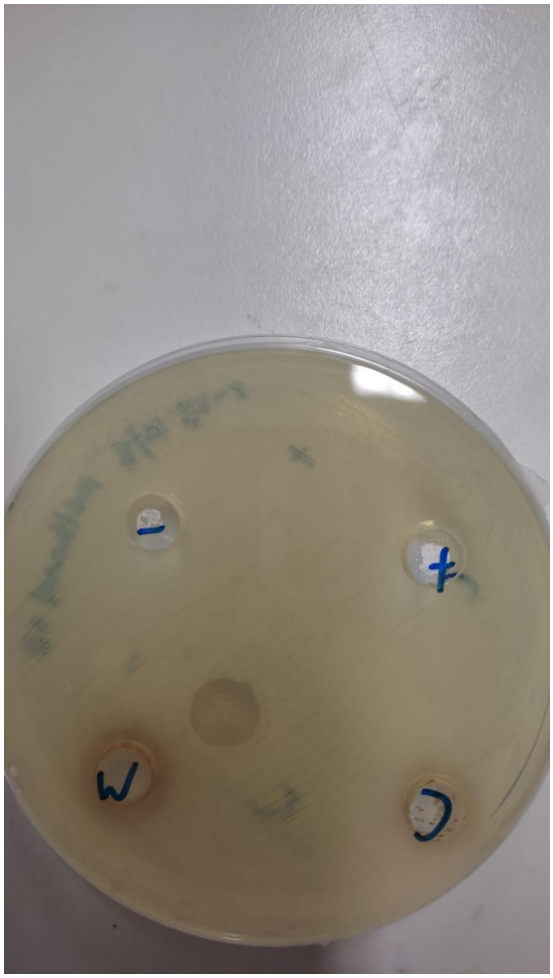

Fig 4: Agar plate shown after conducting well diffusion test of Staphylococcus epidermidis. W represents the walnut extracted using Methanol. C represents the chestnut extracted using Methanol. + represents 10% bleach. - represents sterile water.

(note that the agar plate as shown are the best results in the triplicates we did)

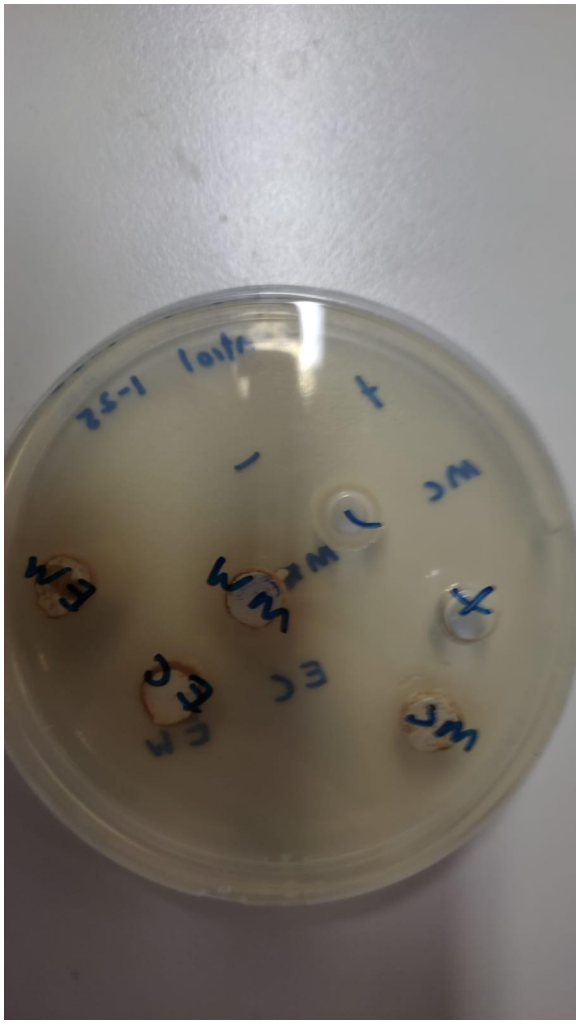

Fig 5: Agar plate shown after conducting well diffusion test on control. WM represents the walnut extracted using Methanol. CM represents the chestnut extracted using Methanol. CM represents the chestnut extracted using methanol. CE represents chestnut extracted using ethanol + represents 10% bleach. - represents sterile water.

	Ethanol *Escherichia coli*	**Methanol *Escherichia coli***	**Ethanol *Staphylococcus epidermidis***	**Methanol *Staphylococcus epidermidis***
Walnut	13.0mm	8.0mm	10.0mm	8.0mm
Chestnut	12.0mm	7.5mm	12.0mm	8.0mm
Sterile water	0.0mm	0.0mm	0.0mm	0.0mm
Bleach	15.5mm	15.5	15.0mm	15.0mm

Table 2: Table showing zones of inhibition (in mm) of each extract for different bacteria, as well as positive and negative control. (1st test with preliminary crude extracts)

	Ethanol *Escherichia coli*	**Methanol *Escherichia coli***	**Ethanol *Staphylococcus epidermidis***	**Methanol *Staphylococcus epidermidis***
Walnut	17.0mm	unobservable	17.0mm	15.0mm
Chestnut	12.0mm	unobservable	12.0mm	unobservable
Sterile water	0.0mm	0.0mm	0.0mm	0.0mm
Bleach	15.0mm	15.0mm	16.0mm	16.0mm

Table 3: Table showing zones of inhibition (in mm) of each extract for different bacteria, as well as positive and negative control. (2nd test with preliminary crude extracts)

	Ethanol *Escherichia coli*	**Methanol *Escherichia coli***	**Ethanol *Staphylococcus epidermidis***	**Methanol *Staphylococcus epidermidis***
Walnut	18.5mm	13.3mm	16.5mm	14.0mm
Chestnut	12.0mm	11.5mm	12.0mm	12.0mm
Sterile water	0.0mm	0.0mm	0.0mm	0.0mm
Bleach	15.5mm	12.8mm	14.5mm	12.5mm

Table 4: Table showing zones of inhibition (in mm) of each extract for different bacteria, as well as positive and negative control. (3rd test with 10g extracts)

As seen from the figures 2, 3, 4, 5 as well as the tables, in all 3 tests, the walnut extracts tended to have a higher zone of inhibition for both bacteria than chestnut extracts. Walnut extracts tended to have an equal or greater zone of inhibition than the bleach control, while chestnut extracts tended to have a smaller zone of inhibition than the bleach control. Throughout all 3 tests, walnut extracts showed slightly better results against *Escherichia Coli* than *Staphylococcus Epidermidis*, while zones of inhibition for chestnut extracts remained largely constant regardless of the bacteria. Furthermore the negative controls, water and the solvents ethanol and methanol did not show any zone of inhibition, meaning that the solvents themselves did not affect the results. By the Kruskal-Wallis test with respect to the negative and positive control, we can determine that the difference between the zone of inhibition of the walnut extracted and the chestnut extracted are indeed significant.

Conclusion

From our tests, we can see that chestnut and walnut shells contain polyphenols, which are strong antioxidants, and both nutshell extracts can inhibit bacterial growth. As such they can be used much similar to the nut kernels, as natural food preservatives to prevent food spoilage through oxidation and both gram-positive and gram-negative bacterial growth. This could be applied in a form such as a gelatin wrapper around raw

food, which would preserve the freshness of the food while being organic and not affecting the food through other chemical processes.

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