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COLOUR REDUCTION AND ANTI-MICROBIAL EVALUATION OF PRETREATED CASHEW LEAVES EXTRACT

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ABSTRACT

Cashew leaves are used traditionally for various health promoting effects including wound healing and diarrhea and could be orally consumed for its effectiveness. Previous research shows that cashew leaves and its bark extracts is rich in tannin and is a potential antimicrobial agent. Extended from these properties, we selected cashew leaves extract as a candidate for potential natural preservative. The extraction method especially using ethanol or other solvent extraction will result in intense colour that will limit its application. The intense green colour of the leaves is due to chlorophyll and become a problem to end product. Various treatments could be used to reduce chlorophyll in the leaves. This study focused on the pretreatment of the cashew leaves in order to minimize the green colour intensity of the extract. Our study shows that pretreatment 3 reduced the green colour intensity significantly. Pretreatment 3: cashew leaves heated in boiling water for 1 minute, immediately cooled in ice-cold water then blotted to dry. The dried leaves then cut into small pieces and floated on the surface of 0.05 M EDTA-2Na, pH 7.0 for 24 hours exposure to the light (5000 lux). The antimicrobial activity of all the extracts was almost similar and was shown to be as effective as methylpareban at concentration as low as 2.5g (v/v). The extract could control the growth of all five main microorganisms as recommended by FDA for cosmetic and bodycare products.

Key Words: Decolourisation, Cashew leaves, Pretreatment, Anti-microbial

1.0 INTRODUCTION

Cashew (*Anacardium occidentale L.*; Anacardiaceae) is a tropical evergreen tree originated from north-east Brazil. Today cashew tree can also be found in India, Vietnam, Africa and South East Asia including Malaysia.

There are many medicinal uses of cashew leaves, bark, and juice from the cashew apple. In Brazil, cashew bark teas were used to stop diarrhea while the caustic shell oil was used to treat skin infections, warts, intestinal worms, and parasitic larvae beneath the skin. Teas and fruit juices from the cashew apple and leaves are known to have antimicrobial, anti-inflammatory, astringent, diuretic, hypoglycemic, and other medicinal properties. The active ingredients in the teas and juices are thought to be tannins, anacardic acid, and cardol. Modern uses of shell oil and fruit juice include facial peels and scalp conditioners and shampoos. The cashew fruit has also been a long time nutritional supplement as it contains up to 5 times more vitamin C than citrus and strawberries. In addition to being delicious and rich in vitamins, it also contains high

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minerals, and other essential nutrients. Volatile compounds present in the fruit include esters, terpenes, and carboxylic acids. The bark and leaves of cashew are a rich source of tannins, a group of plant chemicals with documented biological activity. These tannins, in a 1985 rat study demonstrated anti-inflammatory and astringent effects [1], which may be why cashew is effective in treating diarrhea.

Cashew's antimicrobial properties were first documented in a 1982 in vitro study [2]. In 1999, another study was published indicating it had good in vitro antibacterial activity against E. coli and Pseudomonas [3]. In 1999, researchers reported that cashew fruit exhibited antibacterial activity against the Gram-negative bacterium Helicobacter pylori, which is now considered to cause acute gastritis and stomach ulcers [4]. Its effectiveness against leishmanial ulcers also was documented in two clinical studies [5] [6]. Most recently, a 2001 study reported that a bark extract exhibited in vitro antimicrobial activity against 13 of 15 microorganisms tested [7].

The cashew leaves and bark extract have great potential as an effective antimicrobial agent. The extraction processing of the leaves however will fetch in the green chlorophyll. This dark green coloured extract usually contributed in unwanted greenish colour of end products. In the processing perspective, pretreatment could be useful to eliminate green chlorophyll colour of the leaves.

There are many ways that can be applied to reduce or eliminate green colour of leaves. Boiling the leaves, expose to darkness and soaked in EDTA were reported to responsible for the decolorization of green chloroplasts. Several methods can be used to treat the green leaves before ethanolic extraction in order to reduce green pigment. The scientific literature indicated that a number of unconventional approaches may successfully remove chlorophyll. The recommended approaches are membrane filtration; ion exchange; supercritical fluid extraction and solvent extraction.

Three simple methods of leaves pre-treatment were compared in this study which are:

1. *Pre-treatment 1-* green cashew were soaked in 0.05 M EDTA-2Na, pH 7.0, exposed to light (5000 lux) at room temperature, 25°C for 20 hours.
2. *Pre-treatment 2-* green cashew leaves were soaked in 0.05 M EDTA-2Na, pH 7.0, in darkness and at room temperature, 25°C for 20 hours.
3. *Pre-treatment 3-* green cashew leaves were heated in boiling water for 1 minute and then cooled in ice-cold water. The dried boiled leaves were soaked in 0.05 M EDTA-2Na, pH 7.0 and exposure 24 hours to the light (5000 lux).

2.0 EXPERIMENTAL

2.1 Source of materials

Cashew leaves were collected from Cashew trees grow in the UTM campus. The leaves were wiped clean from dust and residues using wet towel and ready for pretreatment.

Figure 1 Cashew Tree

2.2 Pretreatment Methods

The pre-treatment was done in 3 ways: in lightness, darkness and boiled.

a. Pre-treatment 1-In this method, green cashew were floated on 0.05 M EDTA-2Na, pH 7.0, exposed to light (5000 lux) at room temperature, 25°C for 20 hours.
b. Pre-treatment 2-In this method, green cashew leaves were floated on 0.05 M EDTA-2Na, pH 7.0, in darkness and at room temperature, 25^{0}C for 20 hours.
c. Pre-treatment 3-Green cashew leaves were heated in boiling water for 1 minute and after that cooled in ice-cold water. The boiled leaves were blotted dry, weighed and cut into small pieces and floated on the surface of 0.05 M EDTA-2Na, pH 7.0. After 24 hours exposure to the light (5000 lux), the changed were observed.
d. Control-The leaves floated in water at room temperature for 20 hours were used as control sample. After pretreatment, the leaves were dried at room temperature under shade for 5 to 10 days or oven dried at 45^{0}C for 1 hour before extraction.

2.3 Ethanol extraction

Ethanol extraction was done by using Soxhlet extractor with 95 % (v/v) ethanol as solvent. 25g grounded cashew leaves powder was extracted with 400mL absolute ethanol at 80^{0}C for 18 hours. The mixture was filtered using Whatman No. 1 filter paper and dried using rotary evaporator (BUCHI Rotavapor R-114) at 60^{0}C and 100mbar. The solidified extract was collected and stored in 4^{0}C.

2.4 Moisturiser Based Cream Formulation

The based cream was obtained from Cosmetic Business Unit in Chemical Engineering Pilot Plant (CEPP). The cream then added with different volume/volume concentration (2.5g, 5.0g and 10g) of pretreated cashew leaves extracts. The creams were then tested for its appearance and microbial activity. The composition used is presented in Table 1.

Table 1 The ingredients of based cream and its composition.

ingredient	Composition (gram)							
	A	B	C	D	E	F	G	H
Phase I								
A) Deionised water	74	74	74	74	74	74	74	74
B) EDTA Tetrasodium	1	1	1	1	1	1	1	1
C) Glycerine	1	1	1	1	1	1	1	1
D) Carbopol	1	1	1	1	1	1	1	1
E) Methylparaben	0.1	-	-	-	-	-	-	-
Phase II								
F) Stearic Acid	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
G) Glyceryl Stearate	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
H) Refined Coconut Oil	11	11	11	11	11	11	11	11
Phase III								
I) Propelene Glycol (PG)	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
J) Triethanolamine (TEA)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
K) Cashew Extract	-	2.5	2.5	5.0	10.0	2.5	5.0	10.0

2.5 Microbial Test

Method for isolation of microorganism from cosmetic products is direct colony counts and enrichment culturing. Dilution and plating media that partially inactive preservatives systems commonly found in cosmetic products were used. The isolated microorganisms were identified by routine microbiological methods.

Sample preparation

For the formulated cream, 1 g sample was added into 20 x 150 mm screw-cap tube containing 1 ml sterile Tween 80 plus five to seven 5-mm glass beads (or ten to fifteen 3-mm glass beads). Total contents was mixed with vortex mixer. The total volume was adjusted to 10 ml with sterile Modified Letheen Brooth (MLB) (9 ml) for the 10^{-1} dilution.

Aerobic Plate Count (APC)

Spread plate technique was used to facilitate recognition of different colony types for differential count. Baird-Parker (BP) agar was used to identify *Staphylacoccus* species.

Duplicate sets of Petri dishes containing modified letheen agar (MLA) and BP agar for samples 10^{-1} to 10^{-6} dilutions were labelled. Either 5 or 10 ml of prepared cosmetic preparation were added onto 45ml or 90 ml, respectively, of modified lethen broth (MLB) for 10^{-2} dilution.

Samples were diluted decimally in MLB to obtain complete dilution series from 10^{-1} to 10^{-5} and thoroughly mixed and poured onto surface of solid media in prelabeled petri dishes. Inoculums were spreaded over entire surface with bent glass rod which was first sterilized by dipping in 95% ethanol and quickly flamed to remove the ethanol. Once all the inoculums being absorbed by the medium, plates were inverted and incubated for 48 hour at 30 ± 2^0C (35^0C for BP plates).

All colonies in plate were counted. Results are reported as APC/g (ml) sample. If plates do not contain 25-250 colonies, record dilution and the number of colonies found.

For no colony growing plates, already prepared MLB dilutions with enriching at 30 ± 2^0C for 7 days were used. The enrichments were examined daily for growth. After 7 days of incubation, or once growth was suspected, all enrichments were subcultured onto both MLA and MacConkey agar plates and were incubated for 48 hours at 30 ± 2^0C.

Fungi, yeast and mold plate count

Similar methods were applied for fungi, yeast and mold counting as above but using Potato Dextrose Agar (PDA), containing 40 ppm chlortetracycline. Once spreaded inoculum being absorbed by medium, plates inverted and incubated at 30 ± 2^0C, and observed daily for seven days. The counts obtained on duplicate plates were average, multiply by 10 to allow for the volume plated (0.1 ml), multiply by the dilution factor and was reported as yeast or mold count/g (ml) sample. For fungal enrichments (optional), prepared sample was decimally diluted in Sabouraud's Dextrose Broth and incubated as described above for MLB dilutions. Once growth occurs, enrichments were streaked on Sabouraud's Dextrose Broth, MEA, or PDA.

3.0 RESULTS AND DISCUSSIONS

3.1 The Pre-treatments

The best active ingredients for cosmetics or other products are when a) effective and b) do not spoiled its final appearance. However natural active ingredients based on plant extract usually contain high green pigment due to chlorophyll residues leaked into the extraction solvent. The pretreatment of the green cashew leaves was carried out to reduce the pigmentation of chlorophyll. Three pretreatment methods were used in order to get the least intense green ethanolic extract of cashew leaves. Figure 2 shows the results of the pretreatments applied on the cashew leaves before the ethanolic extraction as described in Experimental section.

The results of three pretreatment methods above (Figure 1) show that Pretreatment 3 gives the best green colour reduction, followed by Pretreatment 1 and the least effective was Pretreatment 2 with respect to control.

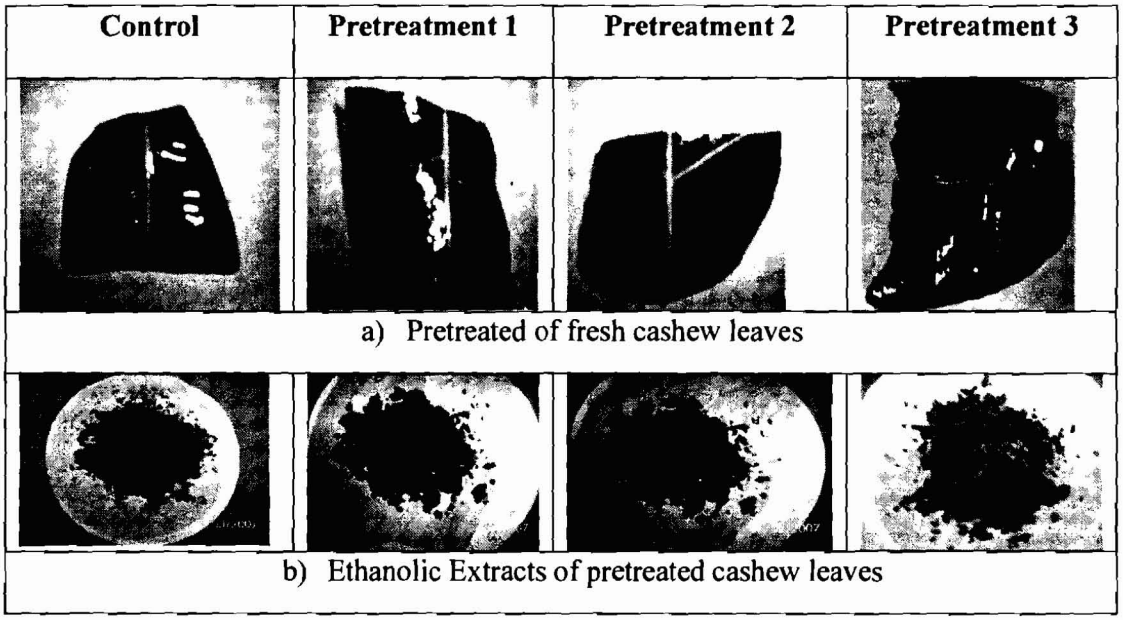

Figure 2 a) The depigmentation of fresh leaves after different pretreatment methods and b) ethanolic extracts of pretreated cashew leaves

3.2 Ethanolic Extraction

After the ethanolic extraction, the grounded pretreated and controlled leaves were transformed to sticky dark green paste as shown in Figure 2.

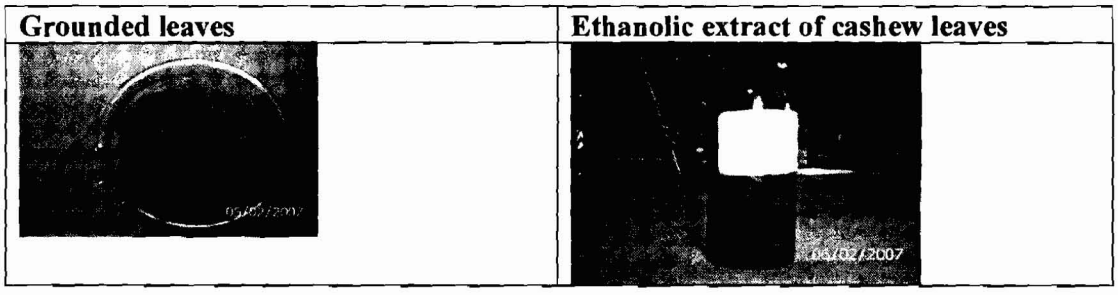

Figure 3 The transformation of grounded cashew leaves to ethanolic extract

3.3 The Appearance Test

The potential application of these pretreated leaves extract was challenged by adding the extracts to one simple moisturiser cream formulation. Pretreatment 1, 3 and control were selected for further analysis. Different amount of pretreated samples' extract were added

to the formulation and were evaluated for its appearance. Figure 3 shows the results of formulated moisturizers using different concentration of pretreated samples' extract. Using 2.5g of each pretreated extracts (2.5%v/v), we found that the formulated creams were lightly coloured which could be acceptable by the costumers. When we increased the amount of extract to 5.0g (v/v) and 10g (v/v) in the formula, it resulted in intense greenish colour of the formulated cream (Figure 4).

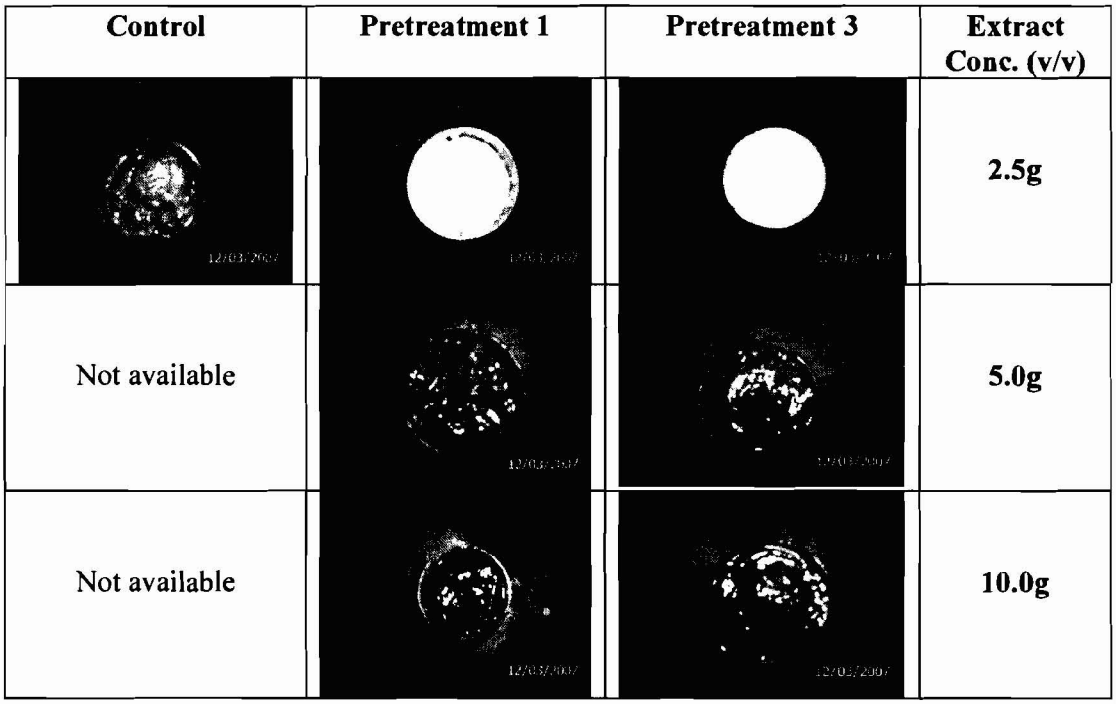

Figure 3 The increase in colour intensity of formulated cream added with 2.5g, 5.0g and 10g of different pretreatment methods of cashew leaves extract.

The appealing colour of cream added with 2.5g indicates the potential of using pretreatment method to reduce green pigmentation of the leaves followed by ethanolic extraction method. Pretreatment 3 gives the least colour tone compared to pretreatment 1 and the control.

Depending on the final application, should this extract to be added as natural preservative agent (as it was previously reported to have antimicrobial activity as discussed in Introduction section) in various types of hand and body cream, the creamish or colourless effect of the formulated cream is recommended. If we are formulating different beauty products like face mask or body scrubs, greenish colour will give authentic and "natural" value to the end products apart from its preservative nature.

3.4 Anti-Microbial Test

The stable and safe formulation is very important in the making of cosmetics and skincare products. The formulated creams were tested for its ability to eliminate microbial contamination.

The results in Table 2 shows that the addition of as low as 2.5g (v/v) of cashew leaves extract inhibited the growth of yeast and mold on PDA plates similar to the

positive control sample using synthetic chemical compound, methylparaben as preservative.

Table 2 Microbial testing on cream samples added with different concentration of pretreated cashew leaves extract

Types of medium agar	Dilution factor	samples							
		A	B	C	D	E	F	G	H
Modified Letheen Agar (MLA)	10^1	-	$3x10^1$	-	-	-	$1x10^1$	$1x10^1$	-
	10^2	-	-	-	-	-	$1x10^2$	-	-
	10^3	-	-	$1x10^3$	-	-	-	-	-
	10^4	-	-	-	-	-	-	-	-
	10^5	-	-	-	-	-	-	-	-
Baird-Paiker (BP)	10^1	$3x10^1$	$2x10^1$	-	-	-	-	$1x10^1$	-
	10^2	-	-	-	-	-	-	-	-
	10^3	-	-	-	-	-	$1x10^3$	$1x10^3$	-
	10^4	-	-	-	-	-	-	-	-
	10^5	-	-	-	-	-	-	-	-
Potato Dextrose Agar (PDA)	10^1	-	-	-	-	-	-	-	-
	10^2	-	-	-	-	-	-	-	-
	10^3	-	-	-	-	-	-	-	-
	10^4	-	-	-	-	-	-	-	-
	10^5	-	-	-	-	-	-	-	-

A = +ve Control (methylparaben)
B = -ve Control (2.5 g cashew leaves extract without pre-treatment)
C = 2.5 g cashew leaves extract with pre-treatment 1
D = 5.0 g cashew leaves extract with pre-treatment 1
E = 10.0 g cashew leaves extract with pre-treatment 1
F = 2.5 g cashew leaves extract with pre-treatment 3
G = 5.0 g cashew leaves extract with pre-treatment 3
H = 10.0 g cashew leaves extract with pre-treatment 3

Overall, the results on gram positive and negative bacterial growth also indicate the potential of using such extract as natural preservative as minimal as 2.5g (v/v). The microbe count obtained in this test is below the minimum standard requirement regulated by Malaysian authority as well as FDA for cosmetic formulation. This finding shows that pretreated ethanolic extract of cashew leaves is a potential antimicrobial agent that can be used as natural preservative in cosmetics and other skincare products.

4.0 CONCLUSION

In conclusion, the finding shows that soaking the leaves in EDTA and exposed to light have successfully reduced the green colour intensity of the leaves ethanolic extract. Furthermore, the pretreatment did not disturb the antimicrobial activity of extracts. Pretreatment 3 exhibited significant colour intensity reduction. The formulated cream using extract from pretreatment 3 also shows least greenish colour which is could be more acceptable in market shelves. The cashew leaves extract also exhibited potential as antimicrobial agent to replace synthetic preservatives in cosmetics formulation. Based on microbial data, formulated cream using cashew leaves extract from Pretreatment 3 complied with authority standard.

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