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ANALYSIS OF PHYTOCHEMICALS IN CASHEW APPLE

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Abstract

The cashew tree (*Anacardium occidentale* L.), is a rich source of phytochemicals with potential health benefits and allergenic properties. In this study, the phytochemical content of the ethanol and ethyl acetate extracts of cashew apple, nut shell, testa, as well as the fermented drink feni was analysed qualitatively and quantitatively through standard tests, thin layer chromatography and FTIR. Ethanolic extracts showed the highest flavonoid and phenolic content. FTIR analysis detected urushiol-like structures in cashew samples and feni, suggesting allergenic potential. None of the extracts demonstrated significant antimicrobial activity, but some had antioxidant and pro-inflammatory activity. This study flags the presence of bioactive compounds in cashew derivatives and their implications in food safety and therapeutic use.

Keywords: Cashew apple**,** feni, urushiols, allergens

1. INTRODUCTION

Phytochemicals are plant-derived compounds responsible for defence, colour, aroma, and flavour, with antioxidant, antimicrobial, anti-inflammatory and anticancer effects. Plants from the Anacardiaceae family which includes mango, cashew, and poison ivy produce urushiol, a toxic compound that causes allergic contact dermatitis but paradoxically, has therapeutic value [1,2,3].
The cashew apple is fleshy, pear-shaped and turns from yellow colour to red upon ripening. Highly edible, it is used in juices, jams, and liquors. It is rich in vitamin C, minerals, and phytochemicals with antibacterial and antioxidant activity [4]. Since cashew apple is known to cause allergic contact dermatitis in some individuals, it was of interest to analyse extracts of the fruit for the presence of urushiols. Also, the fermented cashew apple is distilled into a local drink called feni, which is supposed to have medicinal properties. This study was therefore undertaken to analyse the phytochemicals in cashew apple extracts and feni.

2. MATERIALS AND METHODS

Cashew apples with unripe nuts were collected from a farm in J. Venkatapura, Shidlaghatta taluk, Chikballapur district. Cashew nuts with testa were procured from Madgaon, Goa. The apples, nut shell and testa were crushed in a mortar-pestle and extracted with solvent (water, ethanol and ethyl acetate) overnight. All the extracts were centrifuged

at 5000 rpm for 10 mins at 25°C to separate solids. The supernatants were stored at room temperature in brown bottles to maintain stability and prevent degradation of bioactive compounds. Three brands of commercially available Feni (GSF, PK and F77) were procured from Madgaon, Goa. Two brands of Rhus tox tincture (Schwabe A and SBL) were procured from local retailers of homeopathic medicines in Bengaluru.

Phytochemical tests

50µl of the aqueous/ethanol/ ethyl acetate extracts was used to test for the presence of various phytochemicals (Table 1).

Table 1. Qualitative tests for phytochemicals.

TEST	PROCEDURE	INTERPRETATION
Saponin	50 µL sample + 700 µL distilled water in an Eppendorf tube. Shake for 15 minutes.	Formation of a foam layer: saponins present
Phenols	50 µL sample + 950 µL water in a test tube. Add 1- 2 drops ferric chloride ($FeCl_3$).	Blue, green, red or purple colour: phenols present
Glycoside	50 µL sample + 1 mL water in a test tube Add a few drops of aqueous NaOH	Yellow coloration: glycosides present
Flavonoid	Add 1-5 drops of conc.HCl to 50 µL sample	Immediate development of red colour: flavonoids present
Alkaloid	50 µL sample + 0.2 mL dil.HCl in a test tube. Add 1 mL Meyer's reagent*.	Yellowish coloration: alkaloids present
Tannin	Add 2 mL of 5 % $FeCl_3$ to 50µL sample in a test tube.	Greenish-black precipitate: tannins present
Terpenoid	50 µL sample + 2mL $CHCl_3$ in test tube; 3mL conc. H_2SO_4 added slowly to form a layer	Reddish brown coloration of the interface: terpenoids present
Quinones	1ml extract+1ml conc. H_2SO_4	Red color formed: quinones present
Coumarins	1ml extract + 1ml 10% NaOH	Yellow color formed: coumarins present
Anthra quinones	1ml of extract+ few drops of 10% ammonia	Pink colour precipitate: anthraquinones present
Steroids	1ml of extract+ 1 ml of $CHCl_3$ + few drops of conc. sulphuric acid	Brown/ bluish-brown ring at interface: steroids present

Thin layer chromatography (TLC)

TLC was done on Silica gel 60F254 sheets (Merck) using a solvent system of chloroform: methanol: water (6.5:2.5:1). After the run, the TLC was dried and observed under UV light (254nm) for fluorescent compounds. The TLC was then stained with 0.2% ferric chloride in water. Urushiols, being phenolic in nature, should be seen as dark grey spots with a higher Rf value than flavonoids, which should stain yellow.

Fourier Transform Infra- Red Spectroscopy (FTIR) analysis

FTIR analysis was done at the Dept of PG Chemistry, NMKRV college using a BRUKER 100 62427 FTIR analyzer. The ethanol and ethyl acetate extracts of cashew apple, shell and leaf as well as feni and the Rhus tox tinctures were analysed by FTIR. Interpretation of the FTIR spectra was done by comparing the wave numbers corresponding to the different peaks (https://instanano.com/all/characterization/ftir/ftir-functional-group-search/).

Quantification of polyphenols

Estimation of polyphenols in the extracts and feni was done using Folin Ciocalteau reagent [5]. Dilutions of the extract were incubated with FC reagent in the dark for 30 minutes in an alkaline solution and the intensity of the colour developed was read at 720nm. Gallic acid was used as a standard and polyphenol content was expressed as gallic acid equivalent per ml (GAE/ml).

Antibacterial activity assay

The aqueous and ethanol extracts of cashew apple, cashew nut shell and cashew nut testa was tested for antibacterial activity against *E. coli* (Gram negative) and *S.aureus* (Gram positive) cultures using the agar- well method [6]. 15µL of the extracts was introduced into the well with ampicillin (1mg/mL) as a control.

Antioxidant assay (DPPH method)

3.94g of DPPH was dissolved in 100ml methanol (0.1mM solution) and its absorbance at 517nm determined to be between 0.9 - 1.0. 50µl of extract was added to 3ml of DPPH solution and incubated for 30 mins in the dark. The absorbance of the solution was then measured at 517nm. Total antioxidant activity was calculated as follows:

$$\% \text{ antioxidant activity} = [(A_{control} - A_{sample})/ A_{control}] \times 100$$

Assay for the production of pro-inflammatory molecules (CRP-test)

The pro-inflammatory marker C-reactive protein (CRP) was assayed using anti-CRP-latex test kit (ARK-Ray). 10mL of blood was drawn from a non-allergic person and 700µL was distributed into 12 Eppendorf tubes. 25 µL of the ethanol and ethyl acetate extracts of cashew apple, testa and leaf; 25 µL of feni samples and 25 µL of Rhus tox tincture was added to these tubes and incubated at 37ºC overnight. The following day, the tubes were centrifuged at 4000 rpm for 10 mins. A drop of the supernatant serum was tested for agglutination reaction with a drop of anti-CRP antibody coated latex. CRP was used as a positive control as provided in the kit. The formation of visible agglutination within 2 mins was taken as a positive reaction.

3. RESULTS AND DISCUSSION

It is well known that parts of the cashew tree contain several bioactive compounds. While some information is available on the phytochemicals in cashew apple [4,7] there has been no systematic analysis of phytochemicals either for the apple, or for its fermented product, feni.

Phytochemical analysis of cashew apple and feni

The presence of phytochemicals in the solvent extracts of cashew apple was determined by standard tests (Table 2).

Table 2. Phytochemicals present in aqueous, ethanol and ethyl acetate extracts of cashew apple.

Phytochemical	Aqueous extract	Ethanol extract	Ethyl acetate extract
Alkaloids	-	++	-
Tannins	+	++	-
Saponins	-	++	-
Flavonoids	+	++	++
Quinones	++	+	-
Phenols	-	++	-
Terpenoids	++	++	-
Coumarins	++	++	+
Anthraquinones	-	-	+
Steroids	++	++	-

The aqueous extract retained some tannins, flavonoids, terpenes, coumarins and steroids. Significantly, no phenolics were extracted into the water. Ethyl acetate was the only solvent that showed the presence of anthraquinones. Some flavonoids and coumarins were also extracted into ethyl acetate. The ethanol extract of the apple retained most of the phytochemicals.

This led to the question as to whether feni also contained these phytochemicals, since it is made from fermented cashew apple. However, the phytochemical analysis of the three feni samples – B77, GSF and PK - did not reveal the presence of any phytochemical. One of the feni samples (GSF) was positive for terpenoids (Fig 1).

Fig 1. Reddish brown colouration indicating the presence of terpenoids in the feni sample GSF.

It is possible that the phytochemicals are highly diluted in the feni. Hence, 5ml of feni was evaporated to dryness, reconstituted in ethanol and subjected to the same tests. However, even these samples did not contain detectable amounts of any phytochemicals.

TLC of cashew apple and cashew nut shell

In order to determine the common compounds in cashew apple, aliquots of the extracts were separated by thin layer chromatography using a solvent of chloroform: methanol: water (6.5:2.5:1). The presence of fluorescent compounds (mainly phenolics and coumarins) was determined under UV light. Staining with ferric chloride was also done to determine the presence of other phytochemicals.

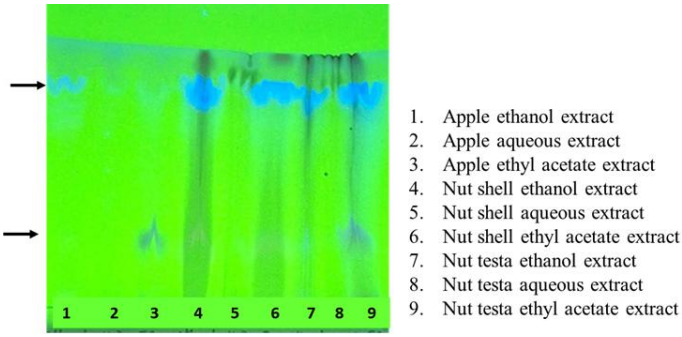

Fig 2. TLC analysis of the solvent extracts of cashew apple, nut shell and testa.

Among the various solvent extracts of cashew apple, ethanol and ethyl acetate extracts showed the presence of fluorescent and phenolic compounds under UV light (upper arrow, Fig 2). The ethyl acetate extract of cashew nut shell and testa also showed the same fluorescence. Interestingly, the ethyl acetate extracts of both cashew apple and cashew nut testa showed an additional band (lower arrow, Fig 2).

TLC of Feni samples with Rhus tox tincture

Once it was established that some common phenolics are present in the cashew apple, nut shell and testa, it was of interest to see if the fermented and distilled liquor "feni" – made from cashew apple - would also show the same phytochemical profile. Along with the feni samples and ethanol extract of cashew apple, Rhus tox tincture was also analysed (Fig 3). Rhus tox is a homoepathic tincture made by extracting the young leaves of poison ivy with an

ethanol-water solvent in the ratio 85:15 [8]. Rhus tox is known to contain minute amounts of urushiols [9]. The last sample to be included in this TLC was an 85:15 ethanol: water extract of the cashew nut shell.

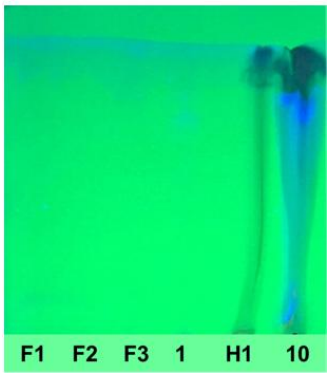

Fig 3. TLC of Feni (F1:B77; F2:PK; F3: GSF), Cashew apple ethanol extract (1), Rhus tox tincture (H1) and cashew nut shell (water + ethanol) (10).

As seen in Fig 3, the feni samples did not show the presence of any detectable phytochemicals either by fluorescence or staining. Both Rhus tox tincture and the nut shell extract showed fluorescence but carried a smear of compounds (Fig 3).

FTIR analysis

Since TLC separation was not conclusive, FTIR analysis of the ethanol and ethyl acetate extracts of cashew apple were carried out as shown in Fig 4.

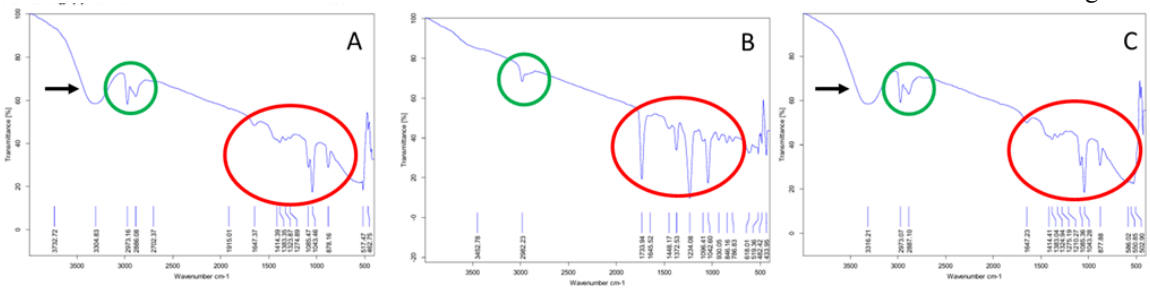

Fig 4. FTIR analysis of the ethanol (A) and ethyl acetate (B) extract of cashew apple and ethanol extract of cashew leaf (C). Common peaks are circled in green, dissimilar peaks are circled red. The ethanol solvent is shown by an arrow in A.

Both the ethanol and ethyl acetate extracts of cashew apple showed a distinctive pair of peaks was obtained at 2973-2886 cm^{-1}. The ethyl acetate extract had several other peaks. It was interesting to note that the ethanolic extract of cashew apple and leaf showed similar profile (compare A and C of Fig 4).

Feni samples showed a similar profile (Fig 5) with twin peaks at 2979 and 2901cm^{-1}.

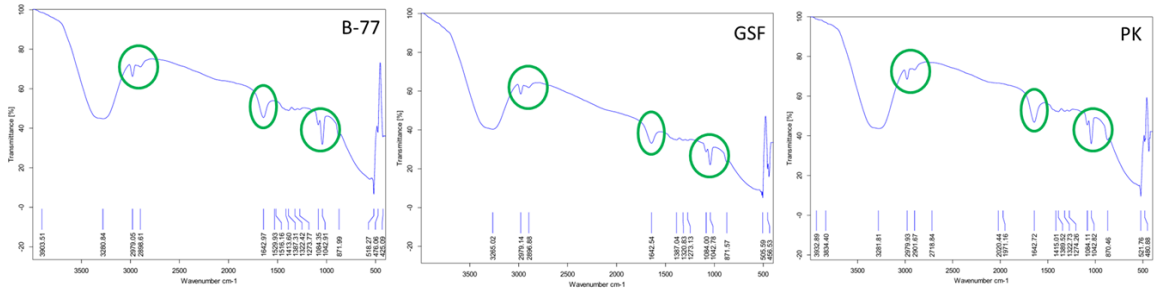

Fig 5. FTIR analysis of the feni samples B77, GSF and PK.

The samples also showed strong peaks at 1642 cm^{-1} and another pair of peaks at 1084 cm^{-1} and 1042 cm^{-1}, corresponding to -C=C- alkene and C-O primary or secondary alcohols, respectively.

Rhus tox tincture (SBL) showed a profile similar to the ethanolic extracts of cashew apple, feni, and cashew nut testa (Fig 7A).

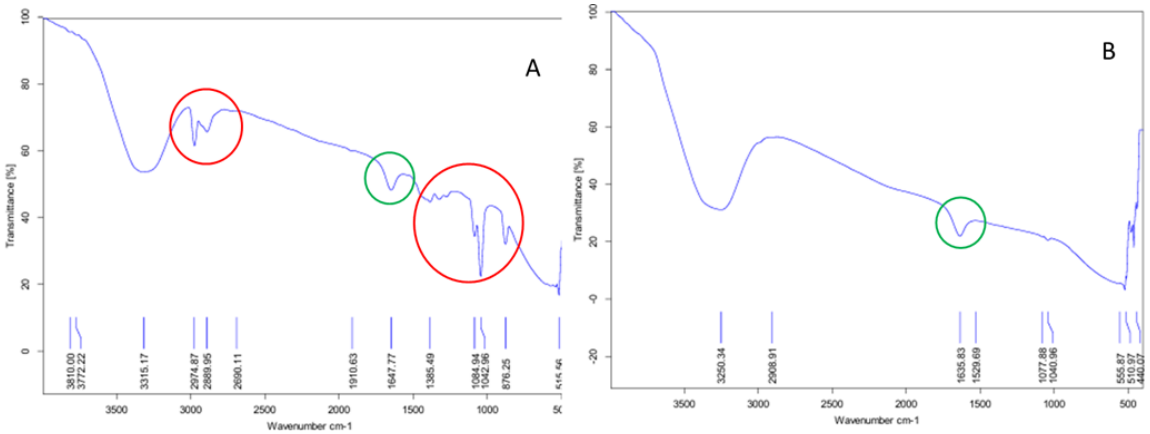

Fig 7. FTIR analysis of Rhus tox tincture from SBL (A) and Schwabe (B). Similar peaks are circled green while dissimilar peaks are circled red.

Rhus tox tincture (Schwabe) showed a profile similar to the ethanol + water extract of cashew nut testa (Fig 7B). This raised the question as to whether these peaks correspond to the same compound.

Possible compound(s) in the samples- Urushiols?

Table 8 shows a collation of the possible groups corresponding to the common peaks shared by the ethanol extract of cashew apple, feni, nut shell, testa and Rhus tox tinctures.

Table 8. Analysis of FTIR spectra from the cashew extracts.

Wave number	Group	Class	Peak
3200-3550	O-H Stretching (H-Bond)	Alcohol (solvent)	Strong, Broad
1600-1650	C=C Stretching	Conjugated alkene	Medium
1395-1440	O-H Bending	Carboxylic acid	Medium
1380-1390	C-H Bending	Aldehyde	Medium
1310-1390	O-H Bending	Phenol	Medium
1266-1342	C-N Stretching	Aromatic Amine	Strong
860-900	C-H Bending	1,2,4-Trisubstituted Benzene 1,3-Disubstituted Benzene	Strong
515-690	C-Br Stretching	Halo compound	Strong
400-600	M-O Stretching	Metal-Oxygen (inorganic)	Strong, Broad

Putting together the bonds that show strong peaks in the FTIR analysis, 1,2, 4- trisubstituted benzene and 1,3-disubstituted benzene appear to be the parent molecules that are present in the extracts as well as the Rhus tox tincture. The addition of the phenolic group(s) and the alkane/ alkenyl groups will then resemble the structure of urushiol (Fig 8).

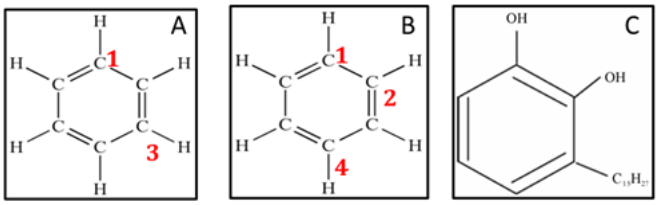

Fig 8. Structures of the substituted benzenes (A and B) and Urushiol (C).

Urushiols from the Anacardiaceae family of plants are chemically distinct yet very similar in structure. They consist of a mixture of various alk(en)yl catechols and/or, alk(en)yl resorcinols and/or alk(en)yl phenols. All urushiols cause contact dermatitis (T-cell mediated delayed type hypersensitivity) but can also confer protective immunity when consumed in minute amounts.

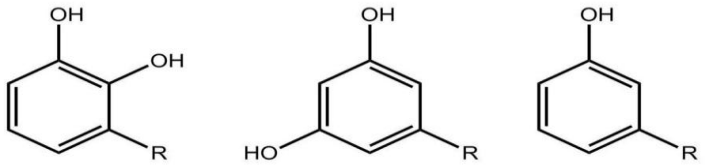

Fig 9. Structures of urushiol congeners – catechols or resorcinols with an R group where R = unsaturated (alkenyl) side chain of 15-17 carbons.

Okahisa *et al*., (2019) [10] have reported that urushiol consists of a variety of isomers of 1,2,3-tri-*o* or 1,3-di-*o* substituted catechols and a long alkyl side chain. FTIR peaks at 992, 1210, 1270, 1460, and 1618 cm^{-1} can be assigned to conjugated triene, aromatic C–O–C, phenolic OH, bending vibration of CH_3 and phenyl ring, respectively.

The molecule present in the extracts contains 1,3- disubstituted benzene, a phenolic group and an alkenyl side chain, similar to urushiol. This suggests that if these molecules are indeed urushiols, they are present in minute quantities (ppm or ppb) and therefore are detected by FTIR but not by TLC**.**

Antimicrobial activity of the cashew extracts

Laxmanaswami and Urooj (2011) [4] had described a significant anti-bacterial activity of cashew apple extract against pathogenic Gram-negative bacteria such as *Salmonella typhimurium* and Gram-positive bacteria like *Streptococcus pyogenes* and *Micrococcus luteus*. However, no significant antibacterial activity was observed against either Gram positive and Gram-negative bacteria or yeast cultures (Fig 10).

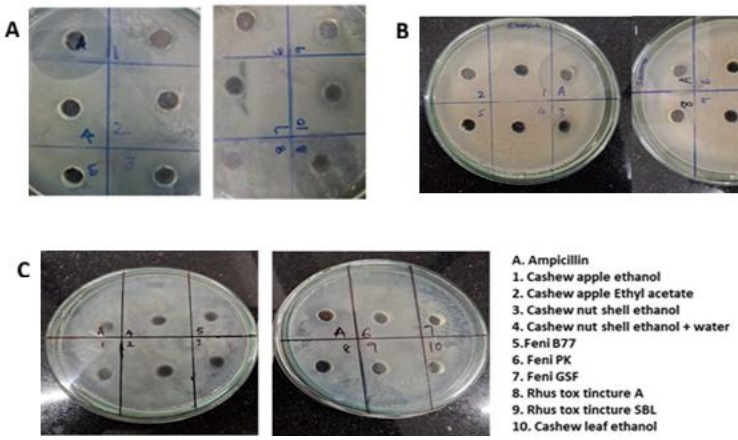

Fig 10. Antimicrobial activity of extracts of cashew apple and nut shell, feni, Rhus tox tincture and leaf ethanol extract against (A) *E. coli* (B) *S. aureus* and (C) *S. cerevisiae*.

Antioxidant activity test

Most phenolics and flavonoids show antioxidant activity, which has significant therapeutic value. The antioxidant activity of the cashew extracts is shown in Fig 11.

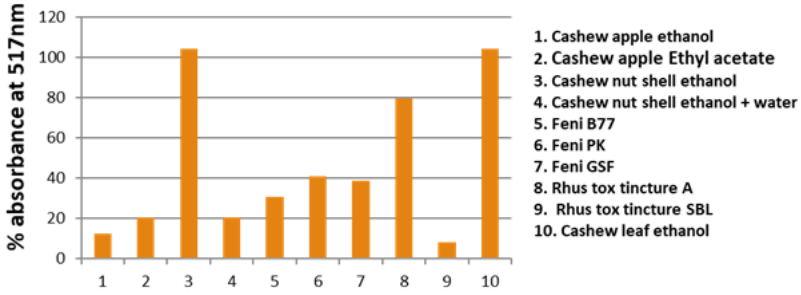

Fig 11. Anti-oxidant activity in cashew extracts, feni and Rhus tox tinctures.

Cashew apple extracts showed low antioxidant activity while the ethanol extract of cashew nut shell and leaf has antioxidant activity approaching complete free radical inhibition. The ethanol-water extract of cashew nut shell showed an 80% decrease in anti-oxidant activity. All three feni samples showed moderate activity, suggesting that there is a loss of antioxidants in the fermentation process. Interestingly, the Rhus tox tinctures displayed consistently strong antioxidant activity, with the SBL preparation showing the highest effect among all tested samples, even surpassing cashew apple extracts.

Quantification of polyphenols in the extracts

Among phytochemicals, polyphenols show the highest antioxidant activity. Hence, the polyphenol content of the cashew apple, shell and testa were determined using gallic acid as a standard.

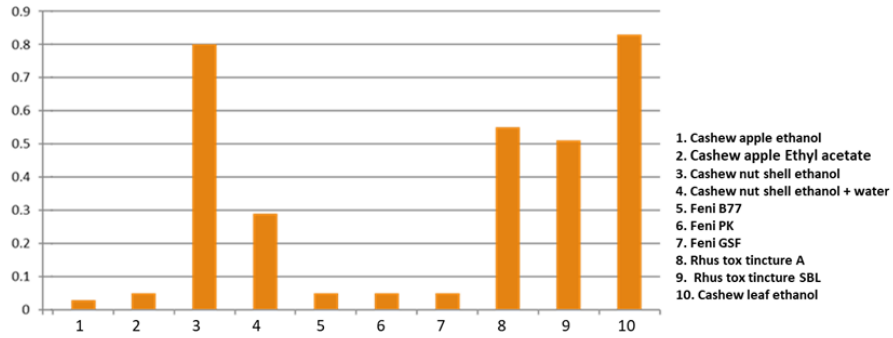

Fig 12. Polyphenol content of the various extracts of cashew apple, nut shell, feni and Rhus tox tincture.

Ethanol extracts of cashew nut testa and leaf showed the highest polyphenol levels, while Rhus tox tinctures had moderate values. Cashew apple extracts and feni samples recorded extremely low polyphenol content. These results correlate well with the antioxidant activity. The only sample that showed a discrepancy was Rhus tox SBL tincture, where the high polyphenol content did not correlate with low antioxidant activity. It may be recalled that this tincture showed a number of additional peaks in the FTIR analysis also. This aspect has to be studied in greater detail.

T- lymphocyte triggered production of pro-inflammatory molecules by extracts

The contact allergic dermatitis caused by the phytochemicals in the Anacardiaceae family are known to involve several pro-inflammatory molecules like TNF-α and IL6. Among these, C-reactive protein (CRP) has also been implicated and can be produced by a subset of peripheral T lymphocytes [11].

To test the possibility of the phytochemicals -especially urushiol and urushiol-like molecules – triggering the production of C-reactive protein, the extracts were incubated with blood from a volunteer with no known history of cashew allergy, at 37ºC overnight. The ethanol extracts of cashew apple and nut shell as well as feni showed a small amount of hemolysis. The other samples (ethanol + water extract of the nut shell and the tinctures) did not show any hemolysis, The blood samples were centrifuged and one drop of the supernatant serum was used for the qualitative test for the presence of C-reactive protein (Fig 12).

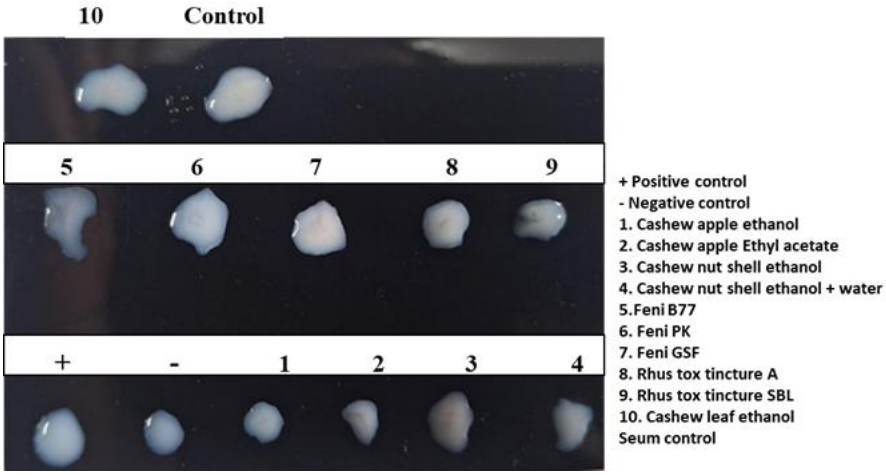

Fig 12. Agglutination of anti-CRP latex by serum incubated with the various extracts. (+) and (–) are the controls given in the test kit; (control) is a serum control without any extract.

Agglutination was noted in sample 3 – Cashew nut shell and in 5 – Feni B77. These were marked as positive, while other samples did not show agglutination (negative). However, the results of this test are not very clear and the test needs to be repeated with a larger number of samples.

4. CONCLUSIONS

The study investigated the phytochemical composition, antioxidant, antimicrobial, and anti-inflammatory activities of cashew apple as well as its fermented product, feni, with a view to identify possible allergenic molecules like urushiols. Extracts of the cashew nut shell and homeopathic Rhus tox tinctures were used for comparison, since they have been

reported to contain urushiols. The polyphenol content and antioxidant activity of cashew apple extracts and feni were much lower than the nut shell. It is possible that in the ripening process, the allergenic molecules move from the apple into the nut shell and serve to protect the nut from being consumed by herbivores. To test this hypothesis, further studies will be done on the cashew apple at various stages of ripening, since this could have a bearing on its use in food products.

5. ACKNOWLEDGEMENTS
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6. STATEMENTS & DECLARATIONS

AI Statement: The authors declare that they have not used generative artificial intelligence, specifically ChatGPT, in the writing of this manuscript and/or in the creation of images, graphics, tables, or their corresponding captions.

Authorship Contribution: Hemashree B: Carrying out the data collection, data curation, and writing the original manuscript. Vinita Balasubramanya: Reviewing the draft and supervision.

Ethical Standards: All the ethical research standards were followed while writing this conceptual paper.

Conflict of Interest: The authors state that they do not have any conflict of interest.

Informed Consent / Ethical Compliance: As this is a conceptual paper, no consent is required.

Human or animal involvement in the article: None

Data Availability: All data included in this research article will be provided on request.

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