Full Length Article

IN-VITRO STUDY OF HAEMOLYTIC ASSAY FOR METHANOLIC EXTRACT OF *Cleome gynandra* PLANT (STINKWEED) AND *Annona squamosa* (CUSTARD APPLE) SEED

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ABSTRACT

Many plants are being used as food additives and also as traditional medicine because of their preservative and curative effects. Various phytochemicals from medicinal plants like polyphenols, flavonoids, and Vitamin C serve as lead compounds in drug design and discovery. These active compounds are used as an alternative medicine to treat diseases like cancer, cardiovascular, Alzheimer's, and Parkinson's disease. Plant extracts can positively affect the red cell membrane and many plants have serious adverse effects, which include induction of haemolytic anaemia. Therefore, many of the commonly used plants need to be evaluated for their potential haemolytic activity and it represents an important starting point in this regard. The haemolytic activity of any compound is the ultimate indicator of general cytotoxicity towards normal healthy cells. Methanolic extract of both Cleome gynandra plant (Stinkweed) and Annona squamosa (Custard apple) seeds were prepared. The haemolytic assay was conducted to check the haemolytic activity of both extracts as well as their combination. The study revealed that Cleome gynandra plant extract had the least haemolytic activity followed by Annona squamosa seed extract and a combination of both extracts. But the haemolytic activity increases when the concentration of all extract increases.

Keywords: Custard apple, Haemolytic Assay, In-vitro, Methanolic Extract, Stinkweed

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INTRODUCTION

India is one of the most important global biodiversity hotspots where evolutionary as well as ecological factors helped to make huge species diversity of medicinal and aromatic plants with various traditional and modern medicinal uses. These plants are being used as food additives and also as traditional medicine because of their

preservative and curative effects (Romano *et al.,* 2021). About 80% of African and Asian populations used these herbal medicines for primary healthcare needs as it is more affordable and accessible, especially in low and middle-income countries (Oyebode *et al.,* 2016). Various phytochemicals from medicinal plants like polyphenols, flavonoids, and vitamin C serve as a lead compounds in drug design and discovery (Kalaivani *et al.,* 2010a). These active compounds are used as an alternative medicine to treat diseases like cancer, cardiovascular, Alzheimer's, and Parkinson's disease (Nakachi *et al.,* 2000; Nwafor *et al.,* 2000; Howes *et al.,* 2003; Houghton and Howes, 2005). They also have antioxidant; anti-inflammatory, antidiarrheal, antimicrobial, Anti-parasitic, Antiviral activities, etc. (McGaw *et al.,* 2000; Elekwa *et al.,* 2003). As most of the plants are having medicinal properties, it is of utmost importance that their efficacy and toxicity risks should be evaluated (Trease and Evans, 2002).

Nowadays, due to the overuse of synthetic drugs, society is going to face great danger and there are also some side effects. With the invention of some advanced scientific techniques, there is a revolution in the herbal medicine industry. However, a lot of processing is needed to develop a drug from natural sources. The toxicity of the active compound plays an important role in drug design. Many plants contain chemical substances that might have a haemolytic or anti-haemolytic effect on human erythrocytes. Several reports indicate that the membranes of human erythrocytes from different blood types have varying stability as determined by the mean corpuscular fragility (Manthey *et al.,* 2001). Plant extracts can positively affect the red cell membrane (De Freitas *et al.,* 2008) and many plants have serious adverse effects, which include induction of haemolytic anaemia. Therefore, many of the commonly used plants need to be evaluated for their potential haemolytic activity and haemolytic activity represents an important starting point in this regard. The haemolytic activity of any compound is the ultimate indicator of general cytotoxicity toward normal healthy cells.

The methanol extract of *Cleome gynandra* (MECG) possesses a very good antioxidant property which may be due to the presence of phenolic substances and flavonoids (Muchuweti *et al.,* 2007). Paul *et al.* (2012) studied that the *Cleome gynandra* leaf extracts showed free radical scavenging capacity which was confirmed by histological examination. Both organic and aqueous extracts of custard apple seed extract exhibit apoptotic properties along with some histolytic activity against tumor cell lines (Pardhasaradhi *et al.* 2004). Dellai *et al.* (2010) also reported the anti-inflammatory activity of these seed extracts.

Therefore before using the Custard apple seed extract, and *Cleome gynandra* plant extract as treatment, this study was carried out to know the haemolytic activity of Custard apple seed extract, *Cleome gynandra* plant extract, and a combination of both extracts.

MATERIALS AND METHODS

Preparation of Extract

The whole plant of *Cleome gynandra Linn*. (Except roots) was collected from a local market Ayurveda shop, in Chennai,

Tamil Nadu, India. The plant specimen was authenticated at the Department of Botany, Presidency College, Chennai, and Tamil Nadu. The *Annona squamosa* (Custard apple) fruits were obtained from the local market, seeds were collected from the fruits. Both plants and custard apple seeds were dried for one month without any contamination and then coarsely pulverized. Then powders were taken in a sterilized beaker, absolute ethanol was added and allowed for magnetic homogenization by stirring at 60-65º C for 15 minutes under 100 rpm. The supernatant was filtered using Whatman #1 filter paper and condensation of the filtrate was done in a rotary evaporator (Heidolph, India) under reduced pressure and vacuum at 40º C and 30 rpm. Then the condensed filtrates were subjected to lyophilization and extract powder was prepared.

Preparation of Erythrocytes Suspension

Four rats were sacrificed by using isoflurane and the blood was collected from direct cardiac puncture. For erythrocyte suspensions, around 10 ml of blood was collected and centrifuged at 3000 rpm for 10 minutes. The plasma was discarded and erythrocytes were washed three times with a sterile Nacl 150mM physiological solution. The final wash was carried out with PBS buffer solution at a pH of 7.4 under the same conditions. The erythrocytes pack obtained (1 ml) was diluted in 49 ml of PBS at pH 7.4, achieving a 1:50 erythrocyte suspension stock (Evans *et al.,* 2013). The washed erythrocytes were stored at 4˚C and used within 6 hours for further studies.

Haemolytic Assay

Triplicates of each test were assembled in 1.5 ml conical bottom tubes. *Cleome gynandra* plant extract, *Annona squamosa* seed extract, and their combination at various concentrations (10, 25, 50, 100, 250, 500, 750, 1000 µg/mL) were taken. The assay was set up to achieve a final volume of 1000 µl in each trial by adding 50µl of a test agent to 950µl of 1: 50 red cell stock suspension. After that, the samples were gently mixed in a shaker and then incubated for 2 hours at 37℃. The samples were then centrifuged at 1200 rpm for 10 min to collect plasma. The plasma was further centrifuged at 12000 rpm for 15 min and the supernatant was collected and 100µl of supernatant was transferred to 96-well plates to obtain the absorbance reading by the spectrophotometer at a wavelength of 540 nm. A 2% Triton-X 100 was used as a positive control and milli-Q water as negative control (Neun *et al.,* 2018).

$$\% \text{ Of Haemolysis} = \frac{(\text{OD of the sample} - \text{OD negative})}{(\text{OD positive} - \text{OD negative})} \times 100$$

Statistical Analysis

The data obtained were evaluated statistically using the Software of Statistical Package for Social Sciences (SPSS 16.0) as per the standard procedure.

RESULTS AND DISCUSSION

In recent days natural products are being shown to be used as tremendous and consistent resources for the development of

new drugs (Kalaivani and Mathew, 2010). These extracts hold promise to be used in different diseases because of their potential sources of antimicrobial, antiviral, and antitumoral agents (Nair *et al.,* 2005). So many plants have already been proven to contain high antioxidant properties as they contain high amounts of phenol and flavonoids (Kalaivani and Mathew, 2009).

The hemolysis assay was presented in Table No. 1 and Fig. 1 and 2. The haemolytic assay result showed that a combination of both extract groups possesses higher haemolytic activity followed by Custard apple seed extract and Cleome gynandra plant extract. Various concentration of all the extract i.e. 10, 25, 50, 100, 250, 500, 750, 1000 µg/mL) were taken to identify the haemolytic activity. As the concentration of extract increased, the haemolytic activity also increased. Thus the extract has shown the dose-dependent haemolytic activity. AT 1000 µg/mL concentration of Custard apple seed extract, Cleome gynandra plant extract and their combination showed haemolysis of 83.00 $\pm$ 0.61, 77.55 $\pm$ 0.73, and 86.12 $\pm$ 0.45 respectively. It was observed that 50, 25, and 10 µg/ml concentration showed no haemolysis in both extracts as well as in the combination of both extracts. But *Cleome gynandra* plant extract showed less haemolysis whereas the combination of both extracts showed more haemolysis as compared to others.

Haemolysis can also happen due to various non-specific mechanisms. Mainly, haemolysis happened due to pore formation in the cell membrane which changes the membrane permeability or it may be due to alteration in the sodium-potassium and calcium-magnesium ATPase activity (Hu *et al.,* 1996). But in some cases, reduced compounds like phenols may be the reason for the oxidation of Haemoglobin producing methemoglobin which finally causes haemolysis (Bukowska and Kowalska, 2004). Measurement of haemolytic activity is important as it is one of the indicators for cytotoxicity. This test has also been employed for the toxicological evaluation of different plants (Gandhi and Cherian, 2000). Mechanical stability of the erythrocytes membrane is a good indicator of various *in-vitro* cytotoxicity (Sharma and Sharma, 2001). Performing a haemolytic assay is important to determine whether a drug is possessing antioxidants and other bioactivities that can be used in pharmacological applications (Kalaivani *et al.,* 2010b).

CONCLUSION

The present study revealed that Cleome gynandra plant extract had least haemolytic activity followed by custard apple seed extract and a combination of both extracts. But the haemolytic activity increases when the concentration of all extract increases. We should be careful about the potential secondary effects generated by the plant therapies as the traditional isn't very useful when the question of evaluating the risk. Along with haemolytic assay, it is very much important to associate all other studies like in-vitro and especially in vivo methods on animal models.

Table 1. Haemolysis percentage of Custard apple seed extract, *Cleome gynandra* plant extract, and their combination

SI No.	Concentration	CAS Extract	CG Extract	Combination
		Haemolysis Percentage		
1	1000 µg/ml	83.00 ± 0.61[b]	77.55 ± 0.73[a]	86.12 ± 0.45[c]
2	750 µg/ml	80.31 ± 0.45[b]	71.73 ± 1.21[a]	82.84 ± 1.26[b]
3	500 µg/ml	75.27 ± 0.45[b]	66.25 ± 0.40[a]	79.48 ± 0.39[c]
4	250 µg/ml	67.32 ± 1.57[b]	53.59 ± 1.59[a]	69.47 ± 1.51[b]
5	100 µg/ml	39.76 ± 2.07[a]	37.22 ± 1.80[a]	46.55 ± 1.57[b]
6	50 µg/ml	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
7	25 µg/ml	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
8	10 µg/ml	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Means bearing different superscripts in a row differ significantly (P<0.05)

** Significant (P≤0.01)

* Significant (P≤0.05)

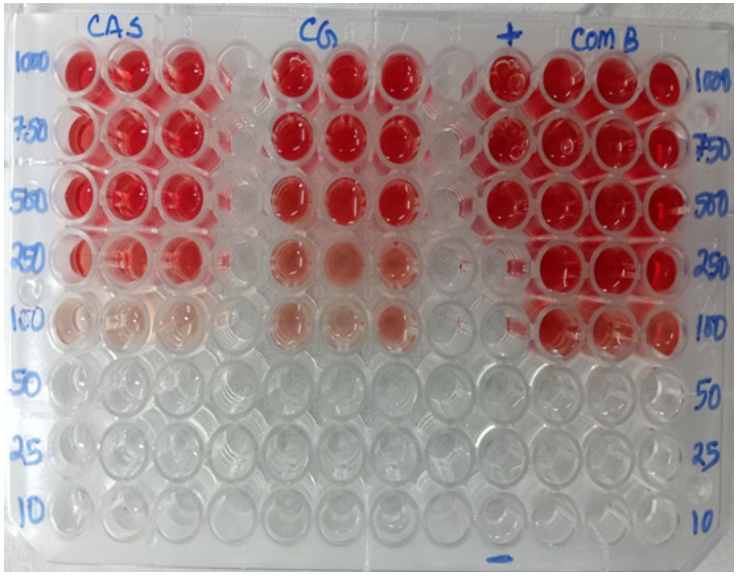

Fig. 1. Haemolysis Assay of methanolic extract of Stinkweed (*Cleome gynandra*) plant, Custard apple (*Annona squamosa*) seed and their combination with different concentration

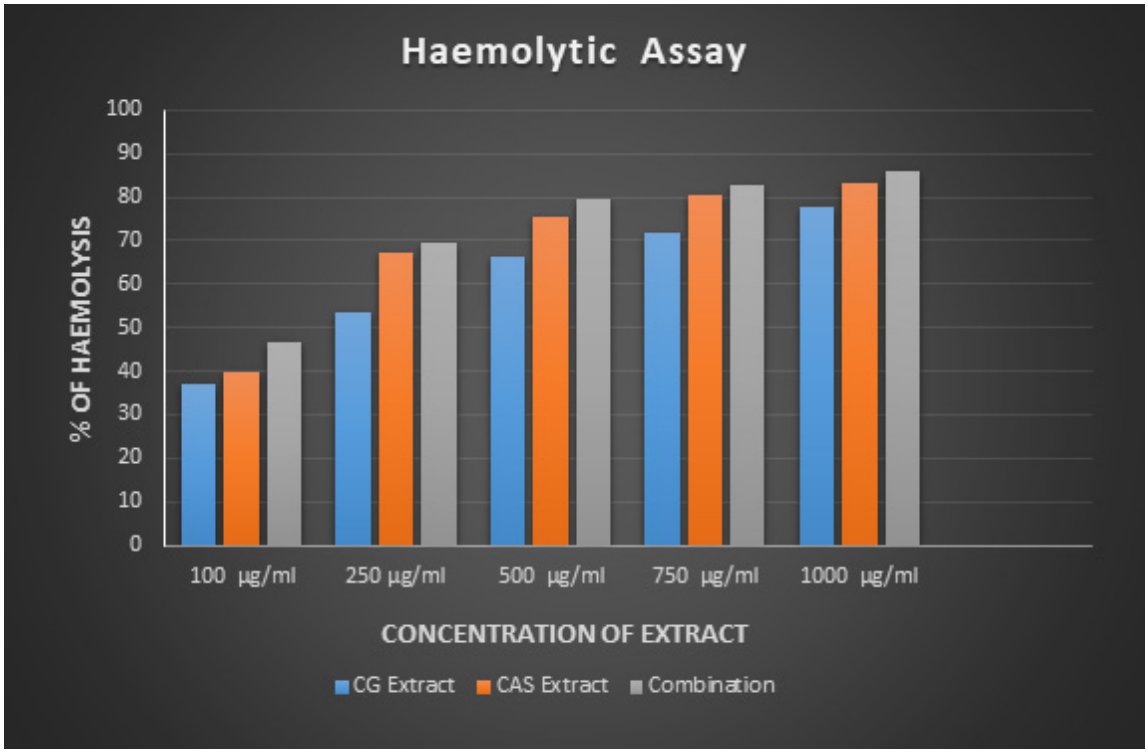

Fig. 2. Graphical representation of Haemolysis Assay of methanolic extract of Stinkweed (*Cleome gynandra*) plant, Custard apple (*Annona squamosa*) seed and their combination with different concentrations

CONFLICT OF INTEREST STATEMENT

All the authors declare that they have no conflict of interest regarding this present research work.

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