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Development and Evaluation of Herbal Formulation for Platelet Augmentation Potential

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Abstract: The present investigation deals with development and evaluation of herbal formulation for platelet augmentation potential. The extraction was carried out by using hydroethanol as a solvent. Per cent yield of hydroethanolic extract of *Tridax procumbens* was found to be 8.65%. Preliminary phytochemical screening showed presence of various secondary metabolites like Alkaloids, Tannins, Amino acids, Flavonoids, Phenols, Steroids, Anthocyanins, Vitamin C and Vitamin A. Total flavonoid content in the extract was found to be 54.22 mg of Rutin/g in hydroethanolic extract. Then animal study was carried out for the estimation of platelet count in Rat model. There were 6 group of animals, each having 6 rats. Group 1 was healthy, Group 2 was for disease control, and remaining 4 groups were for treatment purpose. Two concentrations of extract were given to 2 groups of rats i.e., 100 mg/kg b.wt., and 200 mg/kg b.w. Effective dose was determined by estimating the blood sample for platelet count. Last group was treated with marketed formulation of papaya syrup to compare it with *Tridax procumbens* extract. The results of animal study showed the effectiveness of *Tridax procumbens* by increasing platelet counts of busulfan induced rat model. It showed the recovery of platelet counts as compared to disease control group. The dose of 100 mg is used in the formulation of herbal tablets as per the readings of platelet counts in treatment. The batches of tablet formulation are designed by using Design of Expert (Doe) and evaluation of batches was done. The optimum batch is determined by this design of expert, ANOVA and graphs were taken from this which shows the coded equation of parameters. Dissolution study was carried out of optimized batch by using 0.1M HCl. Then % drug content was calculated by the formula by taking absorbance on spectrophotometer.

Keywords: *Tridax procumbens*, Hydroethanol, Busulfan, Tablet formulation, Optimization by 3^2 factorial designs

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Introduction

Platelets play an important role in clotting and bleeding. Platelets originate from megakaryocytes which are large cells found in the bone marrow. The number of platelets in the blood is referred to as the platelet count and is normally between 1,50,000 to 4,50,000 per microliter of blood. Platelet count less than 1,50,000 are termed thrombocytopenia. Platelet counts greater than 4,50,000 are called thrombocytosis.The function of platelets is very important in clotting system. They circulate in blood vessels and become activated if there is any bleeding or injury in body. Certain chemicals are released from injured blood vessels or other structures that signal platelets to become activated and join other components of system to stop bleeding. When activated, platelets become sticky and adhere to one another and to blood vessel wall at the site of injury to slow down and stop bleeding by plugging up the damaged blood vessel or tissue (hemostasis).

Some of the most common and important causes of thrombocytopenia are outlined below.

(A) *Decreased platelet production*:

Decreased platelet production is usually related to a bone marrow problem (agranulocytosis). These includes viral infections affecting bone marrow for example: parvovirus, Rubella, mumps, chickenpox, hepatitis C, aplastic anemia, chemotherapy drugs and thiazide diuretics, cancers of bone marrow and blood (leukemia) or cancers of lymph nodes (lymphoma), long term alcohol, deficiency of vitamin B12 and folic acid can result in low platelet production by bone marrow.

(B) *Increased platelet destruction or consumption*:

Increased platelet destruction or consumption can be seen a number of medical conditions. They can be divided into immune related and non-immune related causes. Many medications can cause low platelet count by causing immunologic reaction against platelets, called drug-induced thrombocytopenia. Some examples may include: sulfonamide antibiotics, carbamazepine anti-seizure drug, digoxin, quinine, quinidine, acetaminophen, rifampin, and heparin. Some rheumatologic condition, such as systemic lupus erythematosus, Transfusion of blood products, thrombotic thrombocytopenia purpura (TTP) and hemolytic uremic syndrome (HUS) can cause platelet destruction.

(C) *Splenic sequestration*:

Common causes of thrombocytopenia due to splenic enlargement may include advanced liver disease (cirrhosis, for example, from chronic hepatitis B or C) and blood cancers (leukemia or lymphomas) (Tirgar *et al*., 2011).

The present investigation deals with development and evaluation of herbal formulation for platelet augmentation potential. The extraction was carried out by using hydroethanol as a solvent.

Materials and Methods

Materials:

Tridax procumbens plant were collected from local areas of Nagpur. Chemicals used during the present study were ethanol, $NaNO_2$, $AlCl_3$, NaOH, water, methanol, gelatin, corn starch, lactose monohydrate, calcium stearate, calcium carbonate, starch from LOBA Chemie, Mumbai, India.

Extraction of Tridax procumbens leaves:

The Maceration process was used for the extraction. Leaves of *Tridax procumbens* were cleaned and dried. Approximately 185 g of coarse powder of leaves of *Tridax procumbens* were taken into two separate beakers. It was macerated by hydroethanol solution (50:50)

It was stirred continuously for 24 h and then filtered by using Bushner funnel and this operation was repeated for 72 h. Filtrate obtained rotavapour for evaporation of solvent and remaining solvent is evaporated by distillation process. Then filtrate was poured into a petri plate and was put on the water bath for concentrated extract and it was stored in desiccator for further use (Atchade *et al*., 2019).

Table 1: Protocol in animal study

Groups	Treatments		Dose	Animal Model	Post experimentation
I	Control (Healthy group)		-	6	Reuse
II	Busulfan	-	25mg/kg p.o	6	Reuse
III	Busulfan	Vehicle	Water for injection	6	Reuse
IV	Busulfan	Tridax procumbens Extract	100mg/kg p.o	6	Reuse
V	Busulfan	Tridax Procumbens Extract	200mg/kg p.o	6	Reuse
VI	Busulfan	Carica papaya leaves syrup	-	6	Reuse

Determination of total flavonoid content:

The total flavonoid content of *Tridax procumbens* extract was determined by the aluminium chloride colorimetric method. In brief, the hydoalcoholic extract of *Tridax procumbens* was dissolved in 1 ml of methanol, mixed with 4 ml of distilled water and then 0.3 ml of 5% $NaNO_2$ solution; 0.3 ml of 10% $AlCl_3$ solution was added after 5 min of incubation, and the mixture was allowed to stand for 6 min. Then, 2 ml of 1 mol/L NaOH solution were added, and the final volume of the mixture was brought to 10 ml with double-distilled water. The mixture was allowed to stand for 15 min, and absorbance was measured at 510 nm. The total flavonoid content was calculated from a calibration curve, and the result was expressed as mg quercetin equivalent per g dry weight.

Protocol for Animal study (*Jain, 1991*; *Shah*, 2011; Nandini *et al*., *2021*):

Animals had free access to standard pellet diet and water given *ad libitum*.

Group 1: Healthy group

Group 2: Induction of thrombocytopenia in animal model by using Busulfan (Dose: 25 mg/kg b wt. p.o.) for first 3 days (day 1 to day 3), (for group 2 to 6)

Administered animals were left for 4 days to decrease the number of platelet count.

On 8th day 0.5 ml of blood was collected from retro-orbital plexus for estimation of platelet count.

Group 3: Vehicle (water for injection) (for 14 days)

Group 4: *Tridax procumbens* leaves extract (100 mg/kg b wt. p.o.)

Group 5: *Tridax procumbens* leaves extract (200 mg/kg b wt. p.o.)

Group 6: *Carica papaya* leaves syrup.

Platelet count:

After 14 days of treatment, collected 0.5 ml blood from retro-orbital plexus of rats in EDTA K2-anticoagulant tube (using glass capillaries). Platelets counts were performed by automated hematological analyzer or manual microscopic method of counting. Table 1 shows the Protocol for animal study.

Table 2: The various batches of tablet formulation

Sr. no.	T.P. Extract (mg)	Gelatine (mg)	Corn starch (mg)	Lactose monohydrate (mg)	Mg. stearate (mg)	Talc (mg)	Cal. Carbonate (mg)
1	100	35	28	159	7	28	20
2	100	28	28	159	7	28	20
3	100	35	21	159	7	28	20
4	100	21	35	159	7	28	20
5	100	28	28	159	7	28	20
6	100	28	35	159	7	28	20
7	100	21	28	159	7	28	20
8	100	28	21	159	7	28	20
9	100	35	35	159	7	28	20
10	100	21	21	159	7	28	20

Formulation and evaluation of herbal tablet:

Method of preparation:

All the ingredients were weighed accurately and

then uniform mixing was done in the mortar and pestle. Tablet manufacturing was done by using wet granulation method (Sailor *et al*., 2021).

Wet granulation method of tablet was made by mixing extract, Lactose Monohydrate, disintegrating agent corn starch, talc, calcium carbonate, magnesium stearate, and gelatin to form a damp mass which was screened through sieve no. 20 and was dried at 50 ^{0}C. The compositions of batches are shown in Formulation table. All batches were prepared by punching in tablet machine. Table 2 shows the various batches of tablet formulation.

Evaluation of tablet:

Tablet hardness test was done by taking 20 tablets at random, and then placed one by one in the clamp on the hardness tester. Tablet hardness values can be seen on the screen. The terms of good tablet hardness were 4-8 kp.

Twenty tablets were cleaned from dust, weighed (W1), and then put in friability tester, 4 min of rotating at 25 rpm, and then the whole tablet removed, cleaned of dust and reweighing (W2). Losing weight in percentage can be calculated by the formula. Terms friability good is less than 0.8%.

$$\% \text{ Friability tablet} = (W1-W2)/W1 \text{ X } 100$$

Tablet disintegration time was tested by inserting 6 tablets into the basket, and the basket fluctuates in the immersion fluid at a constant frequency rate between 29-32 cycles. If there were no parts of the tablets are left on the screen, except fragments of the coating agent, the tablet could be declared as disintegrated. Disintegration time for the uncoated tablet was no more than 15 min.

Dissolution study:

Dissolution studies were performed for herbal tablet in 900 ml 0.1 M HCl by using USP paddle type II dissolution apparatus at 37$\pm$ 0.5 ℃ at 100 rpm.

% Flavonoid content:

Drug content was determined by dissolving one tablet in 25 ml of methanol in volumetric flask. The solution was filtered through whatmann filter paper. After suitable dilutions total flavonoid content were determined by spectrophoto-metrically Aluminium chloride calorimetric method. Absorbance was recorded by using UV-visible spectrophotometer at 510 nm and the concentration is determined for estimating drug

content.

$$\% \text{ Drug content} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 100$$

ANOVA for animal study:
Graph pad prism:

GraphPad Prism is a highly efficient tool capable of doing all mathematical calculations as well as graphical plotting such as curve fitting. It is not just an ordinary application that makes plotting easier, but also a powerful one that can be utilized even for the superior purposes such as scientific graphics.

Two-way ANOVA:

Two-way ANOVA divides the total variability among values into four components. Prism tabulates the percentage of the variability due to interaction between the row and column factor, the percentage due to the row factor, and the percentage due to the column factor.

Optimization of tablet formulation using 3^2 factorial designs:

Experimental design and optimization of formulation were performed using software. A 3^2 design was applied to the optimization. Two variables and two responses were involved in the experimental design. The variables and their ranges studied are summarized.

The high and low values of each variable were defined based on preliminary experiments. Results obtained for every formula development according to software. The collected experimental data were fitted by a multi linear regression (MLR) model with which several responses can be dealt with simultaneously, to provide an overview of how all the factors affect all the responses. Here, R^2 is the fraction of the variation of the response that can be modeled and Q2 is the fraction of the variation of the response that can be predicted by the model. The relationship between a response y and the variables xi, xj... can be described by a polynome:

$$y = \beta 0 + \beta ixi + \beta jxj + \beta ijxixj + \beta iix2\, i + \beta jjx2\, j + \cdots + E$$

Results and Discussion

Percentage yield:

- YIELD (Wt): 16 g
- % YIELD = W1/W0 X 100

  =16/185 X 100 =8.65
- % YIELD = 8.65 %

Preliminary phytochemical screening:

Table 3 shows the phytochemical screening of *Tridax procumbens* leaves extract.

Total flavonoid content:

Rutin was calculated using the linear regression equation obtained from standard Rutin graph (r^2= 0.9959), y = 0.0055x + 0.0429. Total Flavonoid content was found to be 54.22 mg equivalent to Rutin/g in hydroethanolic extract.

Animal study results:

The platelet count study is illustrated in Table 4 and Figure 1.Firstly, the platelet count shows the normal reading in initial blood sample testing by retro-orbital withdrawal from rat and then after the induction of thrombocytopenia by using busulfan, it shows decrease in platelet counts below the normal range.Then treatment of *Tridax procumbens* extract by oral route for 14 days gives the positive results which shows the increase in platelet count of rat.

ANOVA of Animal study:

If blood platelet count has no effect overall, there is a 0.017% chance of randomly observing an this big (or bigger) in an experiment of this size. The effect is considered extremely significant. Figure 1 shows the graph of effect of treatment in animal's platelet count.

Tablet formulation (*Optimization*):

ANOVA for Quadratic model:

Response 1: *Hardness*

Final Equation in Terms of Coded Factor:

The equation in terms of coded factors can be used

Table 3: Preliminary phytochemical screening in hydroethanolic extract of leaves

PHYTOCONSTITUENTS	HYDROETHANOLIC EXTRACT OF LEAVES
Alkaloids	+
Tannins	+
Amino acids	+
Flavonoids	+
Phenols	+
Steroids	+
Anthocyanins	+
Vitamin C	+
Vitamin A	+

Table 4: The platelet count study in Rat

Group of Animals	Platelet counts in Rat (10^3 / ul)		
(6 Rats in each group)	1st Day	8th Day	21st Day
Group I	689±3.511	198±3.511	705±3.605
Group II	720±2.516	320±2.081	350±3.605
Group III	692±2.516	435±2.516	420±4.509
Group IV	882±2.081	283±2.516	685±3.055
Group V	921±4	333±2.516	690±4.041
Group VI	872±2.516	501±3.214	750±1.527

mean±SD, n=3

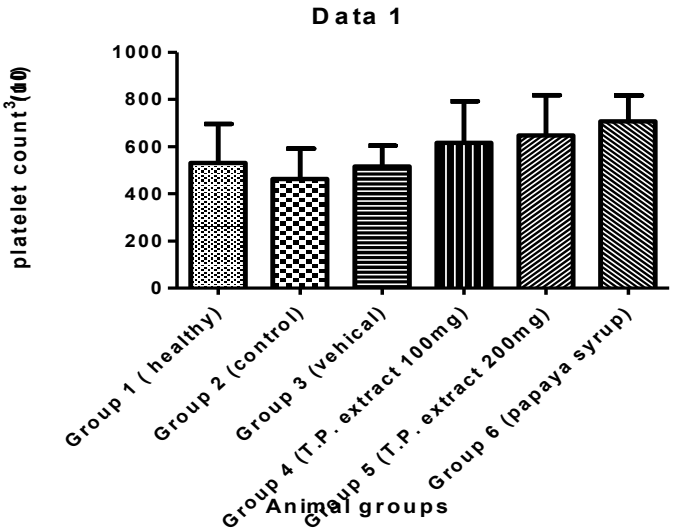

Fig. 1: ANOVA of Animal study.

Table 5: Tablet formulation (Optimization)

		Factor 1	Factor 2	Response 1	Response 2
Std	Run	A:Gelatine	B:Corn starch	Hardness	Disintegration time
		mg	mg	Kp	min
6	1	35	28	6.27	13.8
10	2	28	28	6.1	13.53
3	3	35	21	6.24	13.78
7	4	21	35	5.84	13.45
5	5	28	28	6.1	13.53
8	6	28	35	5.86	13.48
4	7	21	28	6.12	13.67
2	8	28	21	5.68	13.33
9	9	35	35	6.17	13.71
1	10	21	21	5.72	13.38

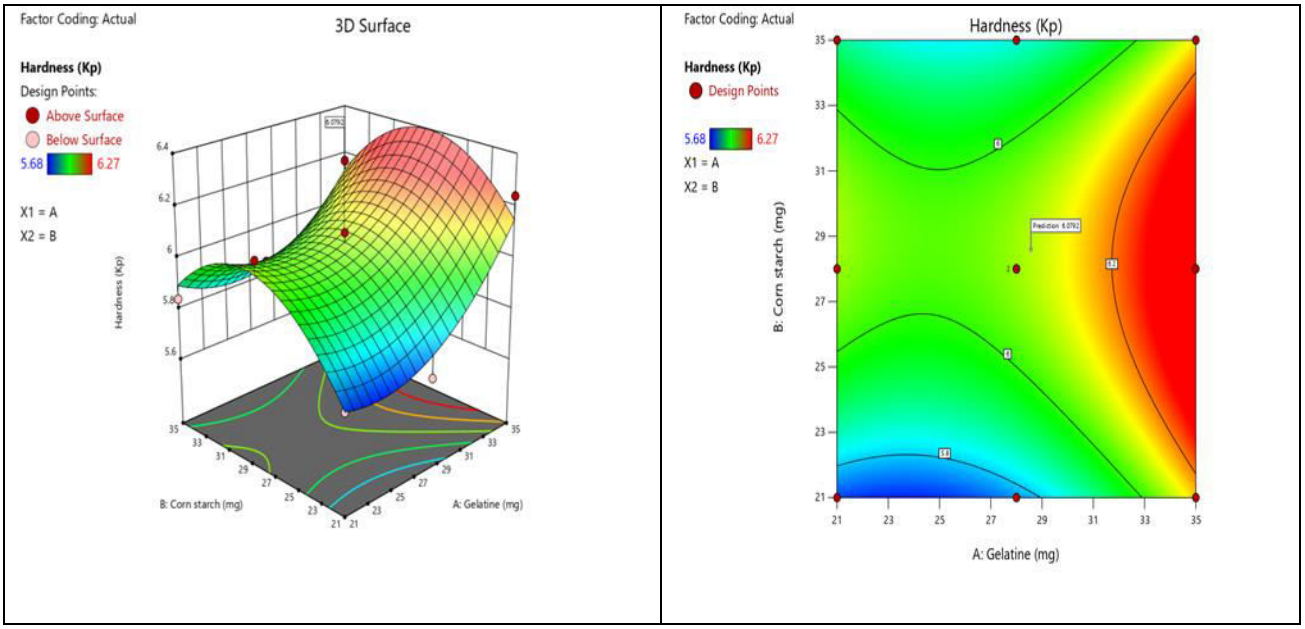

Fig. 2: Contour plot of hardness.

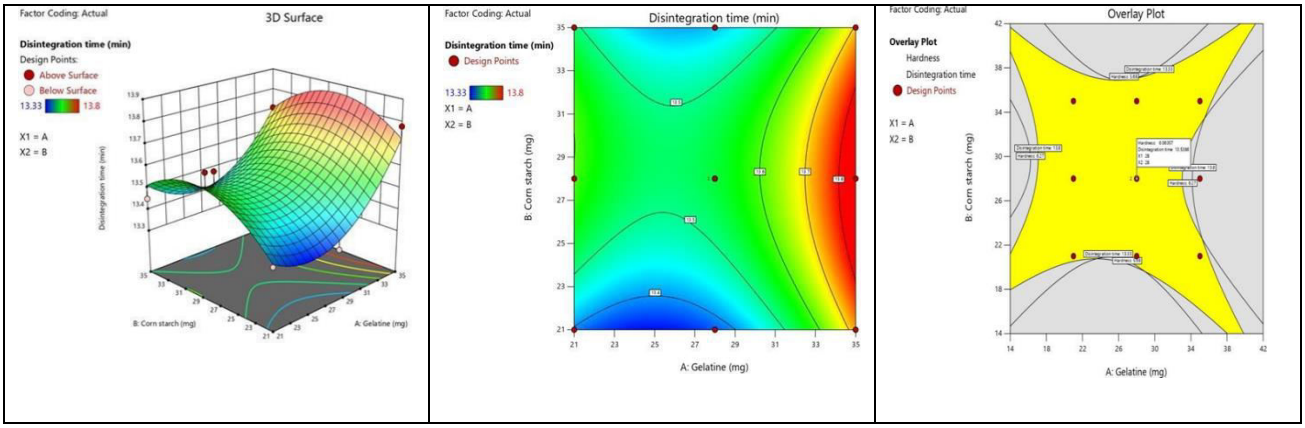

Fig. 3: (a) 3D surface of disintegration time, (b) Contour plot of disintegration time and (c) Overlay plot.

Table 6: Polynomial equation

PARAMETER	EQUATION
Hardness	$Y=6.06+0.1667X_1+0.0383X_2-0.0475X_1X_2+0.1679X^2{}_1-0.2571X^2{}_2$
Disintegration time	$Y=13.54+0.1317X_1+0.0250X_2-0.0350X_1X_2+0.1879X_1{}^2-0.1421X_2{}^2$

Table 7: ANOVA for Quadratic model Response (Hardness and Disintegration time)

Response	Lower limit	Higher limit
Hardness	5.68	6.27
Disintegration time	13.33	13.8

Fig. 4: Tablet formulation.

Table 8: Dissolution study

Time (min.)	Abs.
0	0
15	0.124±0.004
30	0.241±0.003
45	0.357±0.005
60	0.485±0.004
75	0.612±0.004
90	0.63±0.004
105	0.672± 0.003
120	0.754±0.004

to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels are coded as -1. The coded equation is useful for identifying the relative impact of the factors bycomparing the factor coefficients.

Final Equation in Terms of Coded Factors:

The equation in terms of coded factors can be used

to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels are coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.

Herbal Tablet formulation:

Optimized batch of tablet were prepared by wet

granulation method having 28 mg of gelatine as a binder and 28 mg of corn starch as a disintegrating agent which shows the hardness of 0.06 kp and disintegration time of 13.53 min.

Based on the polynomial equation, it appears that the concentration of gelatin has a positive correlation to the tablet hardness response, which means that increasing the concentration of gelatin will increase the hardness of tablet with the corn starch. On the response of friability, if gelatin is combined with corn starch, it reduces brittleness which can increase friability. This is due to the nature of corn starch which is more compressible than the other super disintegrant, so it will form a tablet with a higher hardness and low friability. In this study combination of gelatin as a binder and corn stach as a disintegrant was selected because it produces a tablet with a higher hardness and a low disintegration time. The determination of the optimum formula using design expert program and obtained contour plots of superimposed. The lower and upper limit are determined to get the optimum area.

Dissolution study:

- The dissolution profiles showed that all the formulations released their contents with time over the 60 min period. Within the first 10 min, all the samples tested exhibited relatively fast rates of dissolution, reaching the peak at about the 15th min.
- This corresponded to the period within which disintegration of the tablets were completed which normally precedes complete dissolution.
- After the initial period, the rate generally decreased in nearly the same order for all the tested samples with significant ($P < 0.05$) differences between the fastest and the lowest.
- The tablets prepared by wet granulation showed the highest rate of release.

Conclusion

The hydroethanolic extract of *Tridax procumbens* can increase the platelet counts in the busulfan induced thrombocytopenic SD rats. The herbal tablet formulation development of *Tridax procumbens* hydroethanolic extract can be prepared using Gelatin as a binder and corn starch as a disintegrant using wet granulation method. The optimim formula derived from the design optimization using 3^2 full factorial design is the use of gelatin (28 mg) and corn starch (28 mg), which will generate hardness of 6.06 kp and disintegration time of 13.53 min. From the study it was concluded that *Tridax procumbens* can increase the platelet count in the busulfan induced thrombocytopenia.

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