Antidepressant activity of phenolics rich fraction of *Calotropis gigantea* flower

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Abstract

Calotropis gigantea (Milkweed) is traditionally used to cure bronchial asthma, tuberculosis, spleen disease, diaphoretic and mental disorders. However, no scientific evidence is available to validate the ethnobotanical claim. The present study has been designed to evaluate the antidepressant activity of hydro-alcoholic extract and its major fractions from *C. gigantea* flower. Three different dose regimens were utilized (50, 100 and 200 mg/kg; by gavage) against a conventional antidepressant imipramin (20 mg/kg; I. P.) using tail suspension and forced swimming test as the behavioral animal models. The hydro-alcoholic extract (HACG) and all its major fractions (ethyl acetate soluble: EACG, butanol soluble: BFCG and butanol insoluble: BICG), were able to elicit dose-dependent relation of immobility reduction in the tail suspension and forced swim test in mice. The decrease in immobility time by BFCG at the dose of 200 mg/kg was more significant ($P < 0.001$) compared with negative control and found nearly same as that of imipramin. The antidepressant potential of HACG extracts and its fractions was in decreasing order of BFCG > EACG > HACG > BICG. The increased antidepressant potential of BFCG over other extract and fractions is due to its partial purification achieved by fractionation, which resulted in segregation of secondary metabolites and enrichment of polyphenolic (flavonoid, flavanone and tannin) compounds. These results demonstrated that *C. gigantea* had promising antidepressant effects, which support the traditional belief about the beneficial effects of *C. gigantea* in the nervous system.

Keywords: *Calotropis gigantea, Depression, Flavonoids, Forced swimming test, Polyphenolics, Tail suspension test*

Introduction

Depression is one of the most serious disorders in today's society [1]. It is defined as 'disorders of mood' rather than disturbances of thought or cognition; it may range from a very mild condition, bordering on normality, to severe psychotic depression accompanied by hallucinations and delusions [2]. It is an incapacitating disorder, primarily characterized by a lowering of mood and inhibition of both mental and physical activities. These are two distinct types of depression syndrome, namely unipolar depression and bipolar depression. Unipolar depression, in which mood swings are always in the same direction and bipolar affective disorder, alternates with mania [3]. The World Health Organization predicts that unipolar depression will be the second most prevalent cause of illness-induced disability by 2020 [4]. Recently published data suggest that an estimated 5. 8 % men & 9. 5 % of women experience the depressive episodes in their life time. Today, depression is estimated to affect 450 million people[5]. The World Mental Health Survey conducted in 17 countries found that on average about 1 in 20 people reported having an episode of depression in the previous year [6]. Although the etiology of depression and related illnesses is unclear, it is understood that the combination of factors involving genetic, biochemical, psychological and social causes, all too varying degrees. One of the

major obstacles to clinicians in treating depression with currently available antidepressant is that the therapeutic response develops slowly. Most patients, who will eventually respond to pharmacotherapy, will only begin to show signs of recovery after 2 to 3 weeks of treatment [7]. Indian medicinal plants and their derivatives have been an invaluable source of therapeutic agents to treat various disorders including depression. In recent years, there is increased research on traditional Ayurvedic herbal medicines on the basis of their known effectiveness in the treatment of ailments for which they have been traditionally applied[1]. *Calotropis gigantea* Linn. belonging to family Asclepiadaceae, is a glabrous or hairy, laticiferous shrubs or small trees, commonly known as Swallow-wort or Milkweed. It is found throughout plains, lower hills of India usually near water pounds and grows upto an altitude of 900 m [8]. Traditionally, *C. gigantea* flower is used for bronchial asthma, tuberculosis, fever, pain, pneumonia, spleen disease, diaphoretic, cough, malaria and mental disorders (including epilepsy, convulsions)[9]. By taking into consideration, it is proposed to evaluate the *C. gigantea* flowers for their phytochemicals and antidepressant potential using different behavioral animal models.

Materials and methods

Chemicals and standard drugs

Aluminum trichloride hexahydrate, Folin-Ciocalteu phenol reagent, naringin, gallic acid, rutin (hydrate, min 95%), vanillin and 2, 4-Dinitrophenyl- hydrazine (Analytical grade, S. D. Fine Chemicals Pvt. Ltd. Vadodra, India), diosgenin, catechin (Yucca Enterprises, Mumbai, India) and AR grade chemicals were used.

Plant materials

The fresh flowers of *C. gigantea* were collected during the month of July to August 2013 from the wild region of Nagpur. The flowers were botanically authenticated from the Department of Botany, Rashtrasant Tukadoji Maharaj, Nagpur University, Nagpur. Voucher specimen (9859) has been deposited for future reference. The flowers were lyophilized (Lyodel-00-12, Chennai, India) and the hard brittle masses were crushed to get coarse powder.

Extraction and fractionation

The dried coarse powder was macerated with hydro-alcoholic (ethanolic) solvent (1:1 ratio) for about 48 h. The extract was decanted and marc was again macerated with fresh solvent for about next 5 days. The combined extracts were concentrated using rotary vacuum evaporator and finally lyophilized (HACG; yield: 19. 5 % w/w) (HACG). For fractionation, HACG extract was triturated with silica gel G 60 (1:3), loaded to Soxhlet apparatus and extracted by n-hexane to get n-hexane soluble fraction, (yield: 0. 09 % w/w). The n-hexane insoluble portion was further extracted with chloroform (yield: 0. 27 w/w) then ethyl acetate (EACG; yield: 2. 8 % w/w) and followed by saturated n-butanol (70:30) to yield n-butanol soluble fraction (BFCG; yield: 27. 1 % w/w) and n-butanol insoluble fraction (BICG; yield: 69. 52 % w/w)[10]. The hydro-alcoholic extract (HACG) and these three broad fractions *i.e.* EACG, BFCG and BICG were subjected to phytochemical and pharmacological screening.

Qualitative phytochemical screening

HACG extract and its broad fractions were screened for the presence of polyphenols, saponins, phytosterols, alkaloids, flavonoids, proteins and carbohydrates by using different phytochemical testings [11].

Determination of total polyphenol (TP), flavonoid (FA), flavanone (FO), tannin (TN) and total saponin (TSO) contents

Total polyphenol content was measured with Folin Ciocalteu method using gallic acid as a reference for constructing the standard curve (20-100 µg/ml)

[12]. The results were expressed as mg of gallic acid equivalents (GAE)/g of extract. All determinations were performed in triplicate. Flavonoid content was determined by the aluminium chloride method [13]. Rutin was used as a reference standard (20-100 µg/ml) and results were expressed as mg of rutin equivalents (RE)/g of extract. All determinations were performed in triplicate. The modified 2, 4-dinitrophenylhydrazine (DNPH) method was used for determination of flavanones [14]. Naringin was used as the (500- 2500 µg/ml) standard. The mean of three readings was used and the data expressed as mg of naringin equivalents (NE)/g of extract. The tannin content was determined by using the vanillin hydrochloride method [15]. A calibration curve was constructed using catechin as standard (100-200 µg/ml) which was also treated in a similar manner. The data were expressed as mg of catechin equivalents (CE)/g of extract. The total saponins content was determined by the vanillin-sulfuric acid method [16]. A mean of three readings was utilized; calibration curve was constructed using diosgenin as (20-100 µg/ml) standard and the data were expressed as mg of diosgenin equivalents (DE)/g of extract.

Animals and experimental protocol

Experimental animals

Swiss albino mice (20-25 g) of either sex obtained from animal house of our Institute were used. The animals were fed a standard pellet diet (Hindustan Lever Limited, Hyderabad) and water *ad libitum.* They were maintained in a controlled environment and temperature (22 ± 5 ºC with 12-h of light / dark cycle). All experimental protocols were approved by the Institutional Animal Ethical Committee (21/2014/CPCSEA).

Acute toxicity studies

Mice were divided into test and control groups (n = 6). The test group was given an increasing oral dose (1, 3 and 5 g/kg) of HACG and its fractions. The mice were allowed food and water *ad libitum* and were kept under regular observation for symptoms of mortality and behavioral changes for the period of 48 h [17, 18].

Experimental design

The animals were fasted overnight and divided randomly in fourteen groups (I-XIV) of six mice (n=6) each as follows:

Group I: Received saline as negative control

Group II; Received imipramine in saline (20 mg/kg; I. P.) as positive control

Group III - V; Received HACG - 50, 100 and 200 mg/kg (by gavage)

Group VI - VIII: Received EACG - 50, 100 and 200 mg/kg (by gavage)

Group IX - XI: Received BFCG - 50, 100 and 200 mg/kg

Group XII - XIV: Received BICG - 50, 100 and 200 mg/kg (by gavage)

Tail suspension test (TST)

The different treatments were followed to individual mice in a fixed rotation to ensure a regular distribution of the different treatments over time. Saline, three doses of each extract and fractions were administrated to the mice (by gavage) 60 min prior to the test session. The imipramine was administered intraperitoneally 30 min. prior to the test session. The automated apparatus provides randomization sequences, permit balanced distribution over time and over the different positions in the apparatus. The animal's tail was wrapped around with adhesive tape in a constant position (three quarters of the distance from the base of the tail). Later animal was suspended by passing the suspension hook through the adhesive tape so as the animal hang with its tail in a straight line. The duration of immobility was continuously observed for a period of 8 min and recorded during the last 6 min of observation period [19].

Forced swim test (FST)

Animals were placed in Pyrex cylinders (10 × 45

cm) which were filled with water at 24-25 °C with a 30 cm depth and behaviors were monitored. Similarly the HACG, its major fractions and saline were administrated to the mice (by gavage) 60 min prior to the test protocol. The imipramine was administered intraperitoneally 30 min prior to the test session. The total duration of test was 6 min, after two min, immobility and swimming time was measured during the last 4 min. Immobility was assigned when no additional activity was observed other than that required to keep the animal's head above the water and swimming time assigned when animal did active movement of extremities and circling in the container [19].

Statistical analysis

Data represent mean ± S. E. M. , statistically significant from negative control when * $p < 0.05$ **$p < 0.01$, ***$p < 0.001$, p value calculated by ONE WAY ANOVA followed by Bonferroni's multiple comparison test.

Result and Discussion

Qualitative phytochemical screening

The qualitative phytochemical screening of HACG extract revealed the presence of sterols, carbohydrates, phenolic compounds, flavonoids, saponins and alkaloids. EACG and BFCG fractions have shown the presence of sterols, phenolic compounds, flavonoids, saponins and alkaloids. While, BICG fraction showed the presence of carbohydrates, phenolic compounds, flavonoids, saponins and alkaloids.

Determination of total polyphenol, total flavonoids, tannin and total saponin contents

The total polyphenolic (TP) content (mg/g) was found in a range of 38. 4 to 75. 49 GAE mg/g of extract and the highest content of polyphenolic compounds was found in BFCG fraction (89. 80±0. 06 GAE mg/g of extract) (Table 1). Polyphenol content was determined from linear regression equation of gallic acid and expressed as GAE of extract ($y = 0.010 x + 0.009$, $r^2 = 0.992$) (Figure 1A). The flavones, flavonols and isoflavones formed complexes only

Table 1: Total phenolics (TP), flavonoids (FA), flavanones (FO), total flavonoids (TFA), tannin (TN) and total saponin (TSO) contents in HACG extract and fractions of ***C. gigantea*** flower

Extract/ Fractions	TP (GAE mg/gm of extract)	TN (CE mg/g of extract)	FA (RE mg/g of extract)	FO (NE mg/g of extract)	TFA[a]	TSO (DE mg/g of extract)
HACG extract	75. 49±0. 38	1. 73±0. 01	35. 1±0. 15	18. 0±0. 10	53. 1±0. 03	90. 21±0. 33
EACG fraction	82. 6±0. 02	3. 27±0. 05	30. 6±0. 11	19. 5±0. 14	50. 1±0. 16	40. 0±0. 07
BFCG fraction	89. 8±0. 06	2. 4±0. 08	36±0. 04	30. 5±0. 05	66. 5±0. 20	57. 0±0. 21
BICG fraction	38. 4±0. 12	1. 6±0. 03	12. 33±0. 07	1. 5±0. 01	13. 83±0. 22	66. 72±0. 09

Results are means ± SD of three replicates; where GAE, CE, RE, NE and DE: gallic acid, rutin, naringin, catechin and diosgenin equivalents, respectively; [a] Total flavonoid content (TFA) was determined by adding flavonoid content (FA) with flavanone (FO) content.

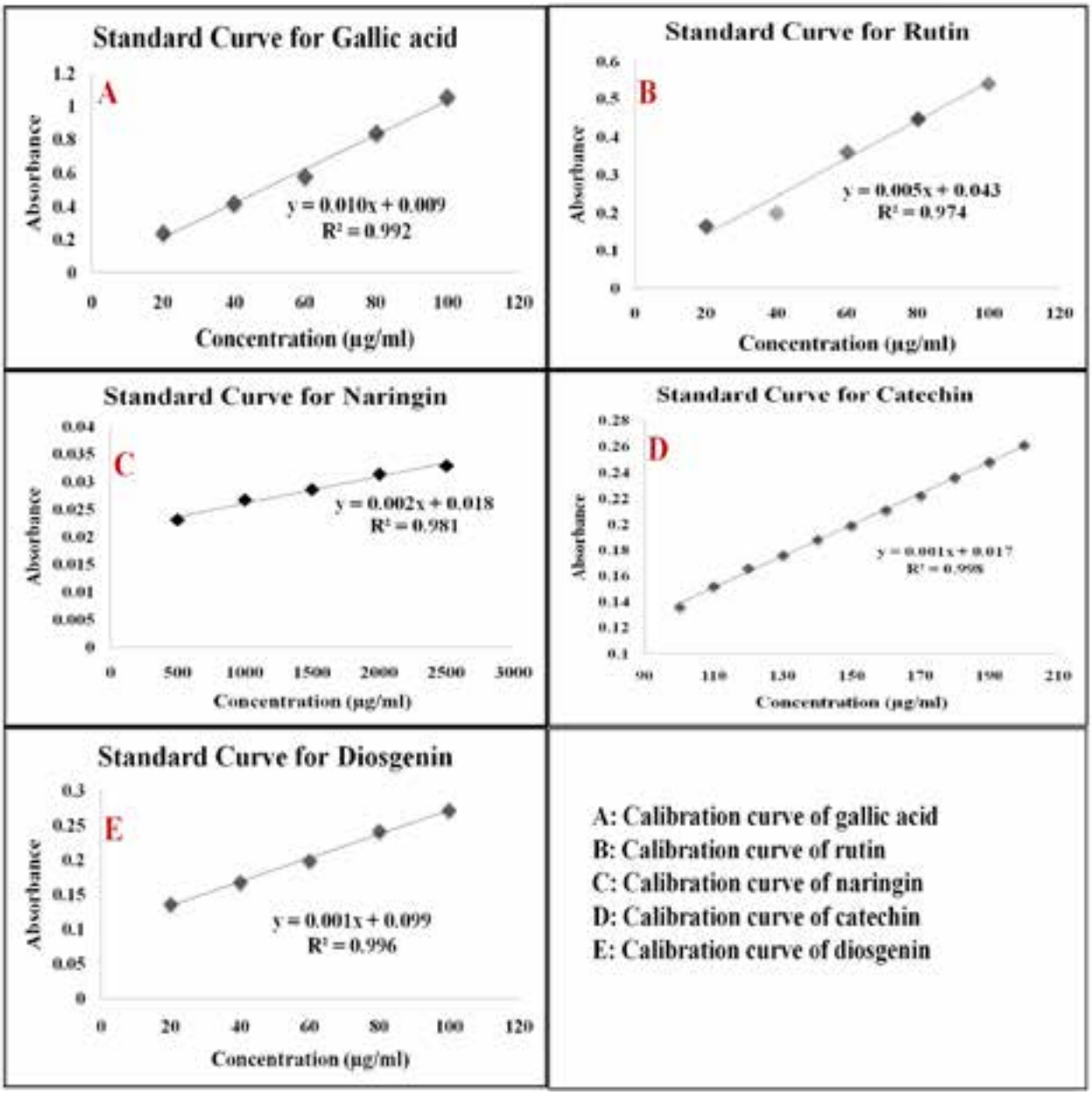
with aluminum chloride, while flavanones strongly reacted only with 2, 4- dinitrophenylhydrazine, so the contents determined by the two methods were added up to obtain the total flavonoid content [20]. The total flavonoid (TFA) content (mg/g) was found in a range of 13. 83 to 66. 50 and the highest content of TFA was found in BFCG fraction 66. 50 mg/g (Table 1). The tannin content (TN) varied from 1. 61 to 3. 27 CE mg/g of extracts (Table 1). Tannin content was ascertained from linear regression equation of catechin (y=0. 001 x + 0. 017, r^2 = 0. 998) (Figure 1D). The total saponin content (TSO) was found in between 40. 0 to 90. 21 DE mg/g of extract. However the highest saponin content was found in HACG extract (90. 21 mg/g) (Table 1). Saponin content was determined from linear regression equation of diosgenin (y = 0. 001x + 0. 099, r^2=0. 996) (Figure 1E).

The extract and all its major fractions, are very complex in their composition, and contain many other polyphenols such as flavanones, isoflavones, phenolic acids and tannins, *etc*. Thus the fractionation of hydro-alcoholic extract HACG with different solvents resulted in separation of important secondary metabolites *i.e.* steroids and other hydrophobic molecules were separated in EACG fraction, while flavonoids and majority of polyphenols were enriched in BFCG fraction, however the saponin were not fully segregated in the fractions. The results were recorded in Table 1.

Antidepressant activity

The prevention and management of stress disorders remains a major clinical problem. Hence it is very important to address these problems and find effective remedies. Though several drugs are available, all are associated with some limitations and there is an urgent need for alternative medications for these disorders [21]. Acute toxicity studies revealed the non-toxic nature of HACG extract and its fractions. There were no lethality or toxic reactions found at any doses selected. In TST and FST animals were subjected to hang and swim respectively later on immobility time was recorded. All the fractions and extract shortened the immobility period in TST, increased the swimming time in FST compared with the negative control and exhibited dose dependent antidepressant activity. Among all the samples, BFCG has shown a potent antidepressant activity with significant (P < 0. 001) decrease in immobility duration 37. 25 sec and 38. 0 sec at a dose of 200 mg/kg compared with negative control in TST and FST respectively (Figure 2 and 3). It was found very close to conventional antidepressant imipramine i. e. 35. 75 sec and 34. 32 sec compared with negative control (P < 0. 001) in TST and FST respectively (Figure 2 and 3). The immobility is thought to reflect either a failure of persistence in escape-directed behavior or the development of passive behavior that disengages the animal from active forms of coping with stressful stimuli[22]. The decrease in immobility time with imipramine, used as a positive control, was however higher. The antidepressant potential of HACG extract and its major fractions was in decreasing order of BFCG > EACG > HACG > BICG. The results obtained for antidepressant activity are in accordance with the results obtained from the quantitative estimation *i.e.* BFCG has shown a potent antidepressant activity which suggests that fractionation of extract has lead to the enrichment of polyphenolic (flavonoid, flavanones and tannins) compounds in BFCG. Results attributed the correlation between the enhanced antidepressant activity and higher polyphenolic compounds.

Figure 1. The calibration curves of reference standards



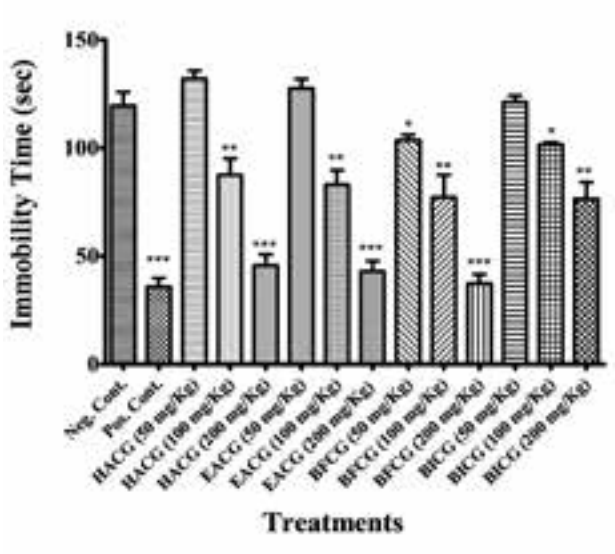


Figure 2. Antidepressant activity of HACG and its major fractions obtained from *C. gigantea* by TST

Values expressed as Mean ± SEM, n=6; * P<0. 05, ** P<0. 01 and *** P< 0. 001 when compared with negative control using ONE WAY ANOVA followed by Bonferroni's multiple comparison test. Where neg. cont. - negative control (received normal saline); pos. cont. - positive control (received imipramine 20 mg/kg); HACG - hydro-alcoholic extract, EACG - ethyl acetate soluble fraction; BFCG - butanol soluble fraction and BICG - butanol insoluble fraction.

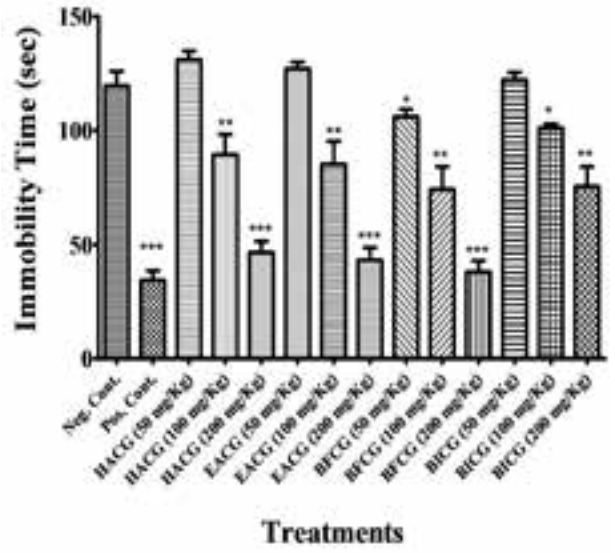


Figure 3. Antidepressant activity of HACG and its major fractions obtained from *C. gigantea* by FST

Values expressed as Mean ± SEM, n=6; * P<0. 05, ** P<0. 01 and *** P< 0. 001 when compared with negative control using ONE WAY ANOVA followed by Bonferroni's multiple comparison test. Where neg. cont. - negative control (received normal saline); pos. cont. - positive control (received imipramine 20 mg/kg); HACG - hydro-alcoholic extract, EACG - ethyl acetate soluble fraction; BFCG - butanol soluble fraction and BICG - butanol insoluble fraction.

Conclusion

From the above evaluations, we conclude that the butanolic fraction of hydro-alcoholic extract from the *Calotropis gigantea* showed significant antidepressant-like effects in TST and FST animal models of depression. Similarly it was enriched with phenolics (flavonoids, flavanones and tannins) compared with all other extracts and fractions. Thus, it is suggested that *C. gigantea* flowers are possibly the potent source of antidepressant agents as it significantly increases the mobility time in TST and swimming time in FST models.

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