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**ATTENUATION OF CISPLATIN-INDUCED EMETOGENESIS BY  
STANDARDIZED *BACOPA MONNIERA* EXTRACTS IN THE PIGEON:  
BEHAVIOURAL AND NEUROCHEMICAL CORRELATIONS**

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Short title: Antiemetic activity of *Bacopa monniera*

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**Abstract:**

Nausea and vomiting are the most distressing and common side effects of cancer chemotherapy which often result in patient non-compliance. In the present study, standardized methanolic fractions of *Bacopa monniera* (*BM*) were evaluated against cisplatin induced emesis in the pigeon in relation to their activity on central and intestinal neurotransmitter levels.

Cisplatin (7.0 mg/kg, i.v.) induced reproducible emesis without lethality in healthy pigeons. The methanolic (10–40 mg/kg), the bacoside rich *n*-butanolic fractions of *B. monniera* (5–20 mg/kg) as well as the antioxidant *N*-(2-Mercaptoprpyonyl) glycine (10 mg/kg) attenuated cisplatin induced emesis by 66.3% ( $P<0.05$ ), 71.6% ( $P<0.001$ ) and 76.5% ( $P<0.001$ ), respectively; where the standard antiemetic, metoclopramide (30 mg/kg), produced a 48.9% reduction ( $P<0.01$ ).

The methanolic and *n*-butanolic fractions of *B. monniera* at all of the doses tested significantly reduced the serotonin concentration ( $P<0.001$ ) in the brain stem and intestine 3 h after cisplatin administration, while at the 18<sup>th</sup> hour, *B. monniera* treatments attenuated not only the dopamine upsurge in the area postrema and brain stem ( $P<0.05-0.001$ ), but also the intestinal 5-HT concentration ( $P<0.01-0.001$ ). *B. monniera* treatments alone did not alter the basal neurotransmitters or their metabolites in the brain areas and intestine.

The prolonged suppressive effect of *BM* treatments on the behavioral signs of cisplatin-induced emesis, the subsequent supportive neural evidence and the safety and tolerability profile suggest that *B. monniera* methanolic and bacoside rich *n*-butanolic fractions might be a valuable adjunct in the treatment of emetogenic chemotherapy and this warrants further study in other models of emesis.

## 1. Introduction:

Nausea and vomiting induced by chemotherapeutic agents like cisplatin are accepted as the most distressing adverse effects of cancer chemotherapy [1,2]. These side effects often result in poor patient compliance and if not satisfactorily controlled, can lead to refusal of treatment [3]. The older classes of antiemetics including antagonists at histamine, dopamine, and muscarinic receptors exhibit only modest effects against chemotherapy emetogenesis. In addition, these antiemetics also possess their own inherent assortment of unpleasant side effects [4].

The development of 5-HT<sub>3</sub> receptor antagonists subsequently revolutionized the treatment of chemotherapy induced vomiting (CIV), and interestingly, an acute (day 1) and delayed (post day 1) biphasic nature of their activities has been perceived. Unfortunately, approximately 10 – 30% of patients are unprotected by 5-HT<sub>3</sub> receptor antagonists [5], the delayed phase of vomiting being the most poorly controlled [6]. Tachykinin NK<sub>1</sub> receptor antagonists, such as aprepitant, have a wider spectrum of antiemetic action than the older classes of drugs, are now being used in combination with 5-HT<sub>3</sub> receptor antagonists and co-administered with glucocorticoids to control emetogenic chemotherapy [7].

Cancer chemotherapy is inevitably damaging to tissues, inducing lipid peroxidation and the generation of free radicals such as hydrogen peroxide, hydroxyl- and superoxide-anions [8,9]. In this regard, there are reports demonstrating the involvement of free radicals in the mediation of cisplatin induced vomiting [10,11]. Generation of free radicals is believed to occur at an early stage of treatment, and it is hypothesized to initiate the release of 5-HT from enterochromaffin cells in the gastrointestinal tract. The subsequent local activation of 5-HT<sub>3</sub> receptors on abdominal vagal afferents is then thought to make a substantial contribution to the mechanism(s) giving rise to CIV [12,13]. Apart from serotonergic receptors [12,14,15],

tachykinin [16,17], cannabinoid [18] and DA receptors [19-21] have also attracted interest as targets for antiemetic efficacy and it is likely that they all play some role in the central control of chemotherapy emetogenesis [18,19,22]. DA D<sub>2</sub> receptors, localized in the limbic system, hypothalamus, amygdala and also in the brain stem emetic circuitry have received particular attention in this respect [23].

The incapacity of any single antiemetic agent to provide universal efficacious control of chemotherapy induced emesis is guiding current research endeavor towards a more wide-ranging mechanistic approach. Indeed, there is evidence for the involvement of several neurotransmitter systems including serotonergic [24,25], dopaminergic [20,21] and neurokininergic [16,26] pathways in emetic neuronal circuitry. In light of this, emesis induced by anticancer drugs is associated with elevated 5-HT levels in the intestinal mucosa as well as the brainstem [27,28] and this is the reason why this neurotransmitter has been considered as one of the primary instigators of chemotherapy induced vomiting for quite some time [29]. This concept is additionally supported by human and animal studies demonstrating increased urinary levels of 5-HIAA [30,31] and 5-HT in intestinal ileal segments.

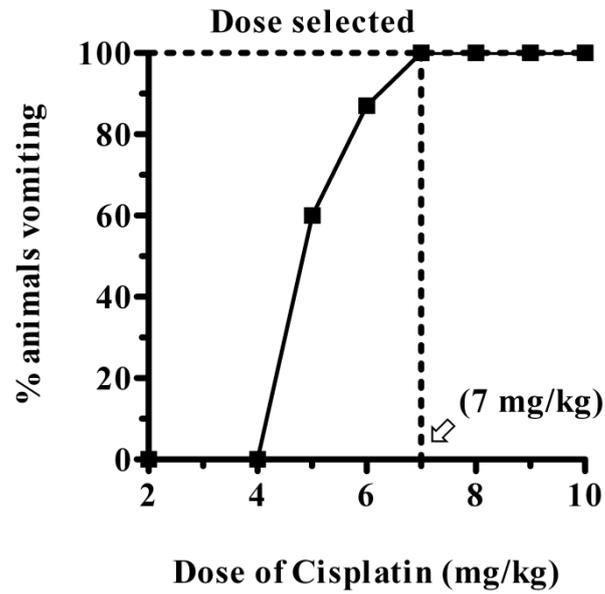
*Bacopa monniera* (Linn.) Pennell [syn *Bacopa monniera* (L) Wettst., *Herpestis monniera*, *Gratiola monniera*) (family - *Scrophulariaceae*), known as “Brahmi” in India and “Jal Neem Booti” in Pakistan is a perennial herb which is found in marshy places around the world including Pakistan[32]. *BM* has a long history of clinical usage in ayurvedic medicine, particularly for the treatment of neuropathic diseases [33,34]. The herb has a number of identified pharmacological properties including an ability to block calcium channels [35], and to prevent DA receptor-mediated hyperactivity in rats [36]. *BM* is also well-known to have antioxidant activity [37] and is protective against aluminum-induced oxidative stress, which is mechanistically similar to the oxidative stress induced by cisplatin [38-41].

In recent years, *BM* has been studied in an attempt to identify its active constituents. Thus, bacoside “A” was shown to be the major constituent comprising a mixture of four compounds, namely bacoside A<sub>3</sub> (Fig 1s) bacopaside II, (Fig 2s) bacosaponin C (Fig 2s), and jujubogenin isomer of bacosaponin C [42]. Since *BM* possesses strong antioxidant activity, and inhibitory effects on hyperactivity mediated by DA receptors [43], we hypothesized that standardized extracts of the herb may have antiemetic properties. The present studies were designed, therefore, to investigate any antiemetic potential of the methanolic fraction of *BM* (BM-MetFr), and the bacopaside rich n-butanolic fraction (BM-ButFr), against the cisplatin-induced retching and vomiting paradigm in the pigeon. In conjunction with this work, concentrations of neurotransmitters and their metabolites were evaluated in the brain and intestine in order to expose any correlative changes with antiemetogenesis.

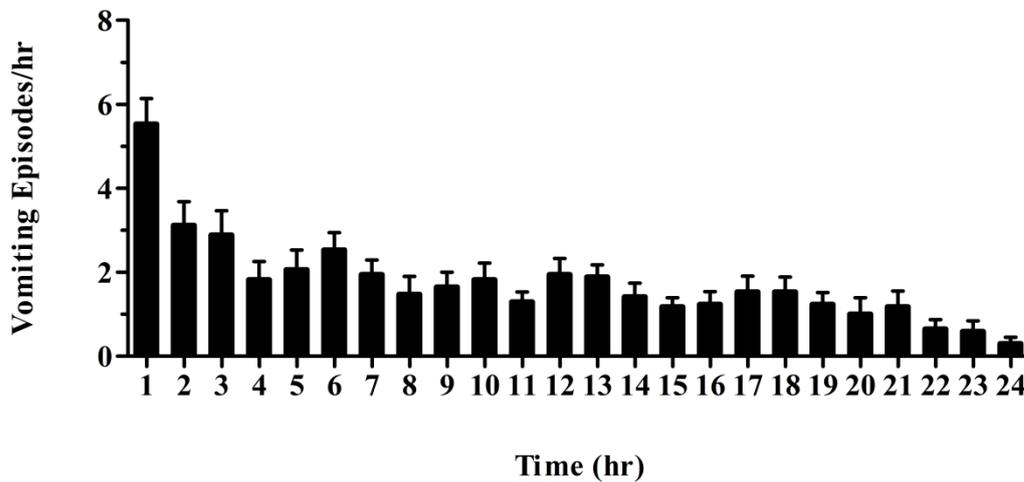
### **3. Results:**

Cisplatin induced vomiting at doses as low as 5.0 mg/kg, but the response was only seen in 60 % of pigeons. Cisplatin (7.0 mg/kg) induced a response in all animals tested which comprised approximately 43 episodes following a mean latency of ~ 67 min (Figure 1A). Increasing the dose further augmented the number of vomiting episodes. Regardless of the dose, all responding animals appeared to vomit within the first two hours after cisplatin administration, with the most intense period occurring around the first hour (Figure 1B). There were no mortalities during 24 h of observation period.

**A:** Dose-response relationship of cisplatin:



**B.** Time profile of cisplatin induced vomiting up to 24 hr in the pigeon



**Figure 1.** (A) Dose-response relationship for cisplatin (2.0 - 10 mg/kg) to induce vomiting in pigeons and (B) profile of cisplatin (7.0 mg/kg i.v.) induced vomiting during a 24 hr observation period. Data are represented by  $\pm$  s.e.m. (n = 17).

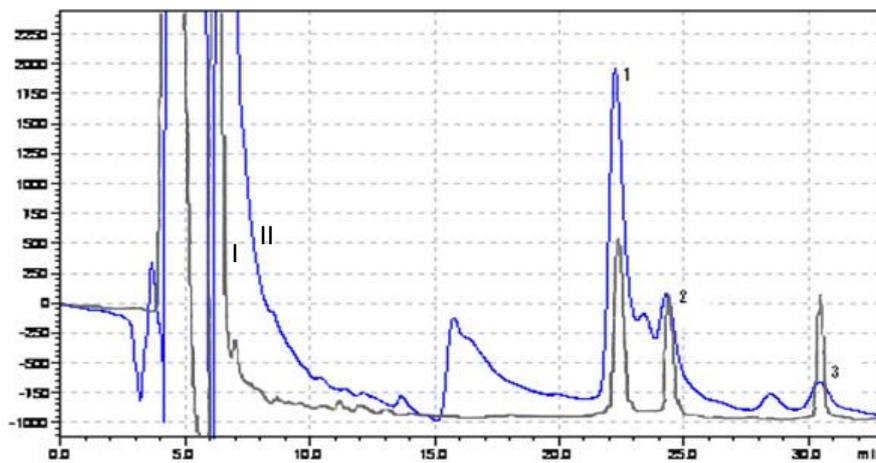
A phytochemical investigation of crude BM-MetFr revealed the presence of saponins, flavonoids, tannins and triterpenoids, and the absence of alkaloids and glycosides (Table 1s).

**Table 1(s).** Phytochemical screening of BM-MetFr

Sample No	Test	Observation	Result
1	500 mg of BM-MetFr + 2 mL acetic acid + 1 drop FeCl <sub>3</sub> + 1 mL H <sub>2</sub> SO <sub>4</sub>	Absence of brown ring color at interphase	Glycosides absent
2	500 mg BM-MetFr + 5 mL chloroform → mixed for 5 minutes → 10% ammonia solution was added	Absence of light green color in upper ammonia layer	Glycosides absent
3	300 mg BM-MetFr + 5 mL chloroform → warmed for 30 minutes → sulphuric acid was added	Dark red color in lower layer	Triterpenoids present
4	Aqueous aliquot of BM-MetFr + Ferric chloride reagent	Green black color	Tannins present
5	0.1 g of BM-MetFr + 10 mL distilled water → Filtered → 1 mL filtrate + ferric chloride reagent	Green black color	Tannins present
6	100mg BM-MetFr + 5 mL ethanol → Filter → 1 mL filtrate + 0.5N KOH	Yellowish color	Flavonoids present
7	300 mg BM-MetFr + 5 mL Distilled water → boil for 2 minutes and cool → vigorous shaking	2.5cm (froth length)	Saponins present
8	0.2 mg BM-MetFr + 5 mL normal saline → filtration → 1 mL filtrate + 4 mL of blood → centrifuge for 5 minutes	Severe hemolysis	Saponins present
9	0.3 g semisolid BM-MetFr + 2M HCl + Amyl alcohol → examine the alcoholic layer	No pink color	Alkaloids absent
10	0.1 g Semisolid BM-MetFr + 10% HCl → filtration → 1 mL filtrate + dragendorff's reagent	No ppt or turbidity	Alkaloids absent

HPLC - UV analysis of the *BM* methanol fraction provided finger prints for the presence of bacoside “A” major components including bacoside A<sub>3</sub>, bacoside II and bacosaponin “C”. The total run time was 33 minutes at a flow rate of 0.6 mL using a wavelength of 205 nm (Figure 2). The peaks and retention times were first confirmed using standards of the respective bacosides. Our results indicated the presence of these bacosides in concentrations of  $24 \pm 1.1 \mu\text{g}/\text{mg}$ ,  $4.76 \pm 0.03 \mu\text{g}/\text{mg}$ ,  $1.23 \pm 0.01 \mu\text{g}/\text{mg}$  ( $n = 3$ ) for bacoside A<sub>3</sub>, bacoside II and bacosaponin C, respectively. Likewise, the total concentration of bacoside “A” major components in BM-MetFr was  $29.99 \pm 2.1 \mu\text{g}/\text{mg}$  of extract.

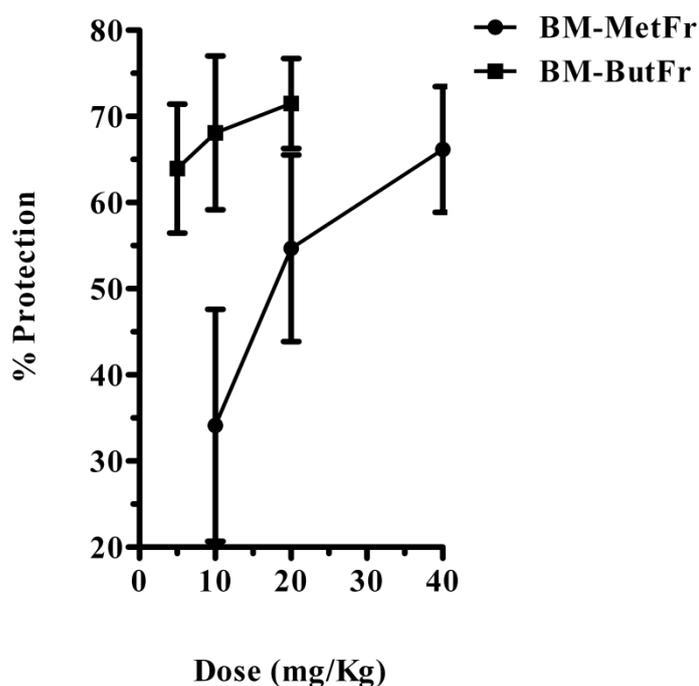
HPLC analysis of the *BM* n-butanol fraction revealed that it was the bacoside rich fraction which contained the bacoside “A” major components: bacoside A<sub>3</sub>, bacoside II and bacosaponin C in concentrations of  $57.91 \pm 3.2 \mu\text{g}/\text{mg}$ ,  $40.60 \pm 0.9 \mu\text{g}/\text{mg}$ , and  $17.23 \pm 1.7 \mu\text{g}/\text{mg}$  ( $n = 3$ ), respectively. Similarly, the total concentration of the three major components within bacoside “A” was  $115.74 \pm 3.9 \mu\text{g}/\text{mg}$  in the n-butanol fraction or  $38.37 \pm 0.7 \mu\text{g}/\text{gm}$  of the dry powder. These values closely correlated with the values reported previously by Rauf and others [44].



**Figure 2.** HPLC chromatogram showing peaks of bacoside “A” major components bacoside A<sub>3</sub> (1), bacoside II (2) and bacosaponin C (3) of standard (I) BM-MrtFr (II) fractions.

In preliminary studies, cisplatin was selected at the dose of 7.0 mg/kg to evaluate the antiemetic potential of *BM* fractions. In these experiments, cisplatin induced vomiting with a latency of ~ 67 min and comprising a total of 43 episodes. The *BM* methanolic fraction (10, 20 and 40 mg/kg) dose dependently reduced cisplatin induced vomiting (Fig. 3), with the highest dose delaying the onset of vomiting by approximately 194 min and reducing the total number of episodes to  $13.8 \pm 2.9$  (66.3 % protection) ( $P < 0.05$ ; Table. 1) during the 24 hr observation period. The antiemetic action appeared to last for up to 16 hr (Fig. 4 B).

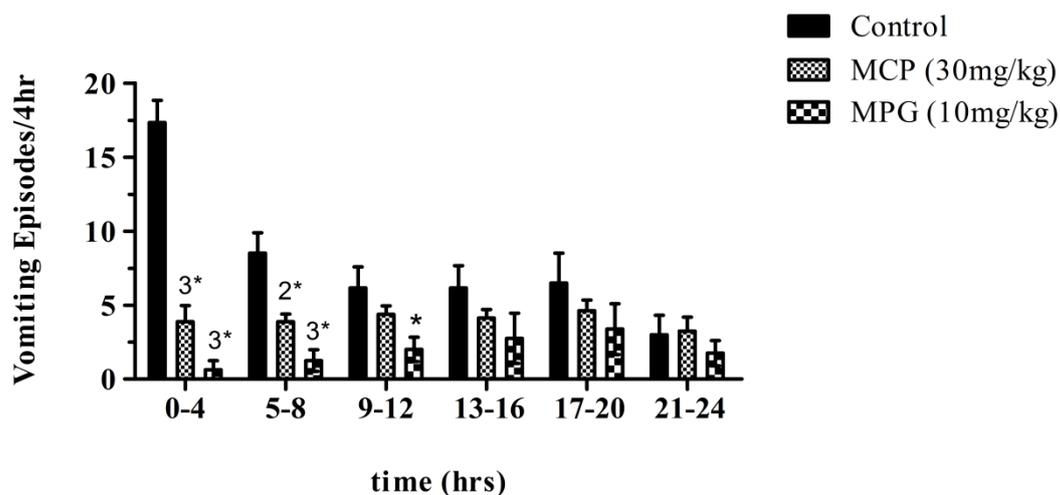
Similarly, the *BM* n-butanolic fraction (5 - 20 mg/kg) reduced cisplatin induced vomiting to  $12 \pm 2.2$  episodes (71.6 % protection) and delayed the onset by approximately 67 min. (Table 1) Moreover, the antiemetic action was evident for up to 24 hr in animals treated with 5.0 and 10 mg/kg ( $P < 0.001$ ; Fig. 4 C). BM-ButFr (10 mg) proved to be superior as it suppressed the response at least in one animal completely. MCP (30 mg/kg) delayed the onset of vomiting by 130 min and reduced vomiting during the 24 hr observation period by 48.9 % ( $P < 0.001$ ). Furthermore, unlike BM-MetFr and BM-ButFr, standard metoclopramide (MCP; 30 mg/kg) was only found to be significantly effective to reduce vomiting during the first 8 hr period (Fig. 4A).



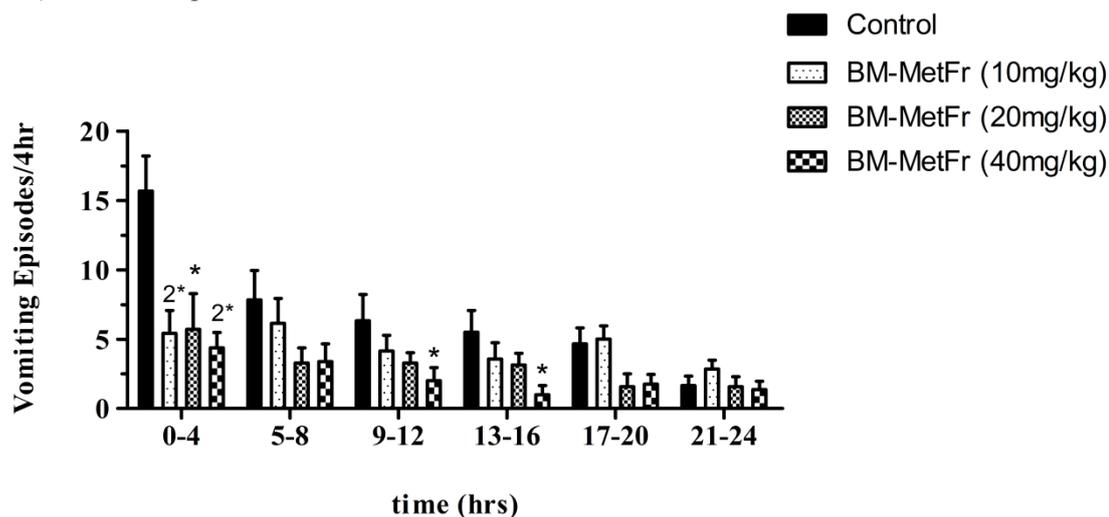
**Figure 3.** Dose response relationship of *Bacopa monniera* methanolic fraction (BM-MetFr) & n-butanolic fraction (BM-ButFr) expressed as % protection against cisplatin induced emesis in pigeons. Data represents the mean  $\pm$  s.e.m ( $n = 7 - 8$ ).

The antioxidant N-(2-Mercaptopropionyl) glycine (MPG; 10 mg/kg) attenuated cisplatin induced vomiting to 76.5 % ( $P < 0.001$ , Table 1 2) and delayed the onset of vomiting by 347 mins but the vomiting suppression was observed up to 12 h (Fig. 4 A).

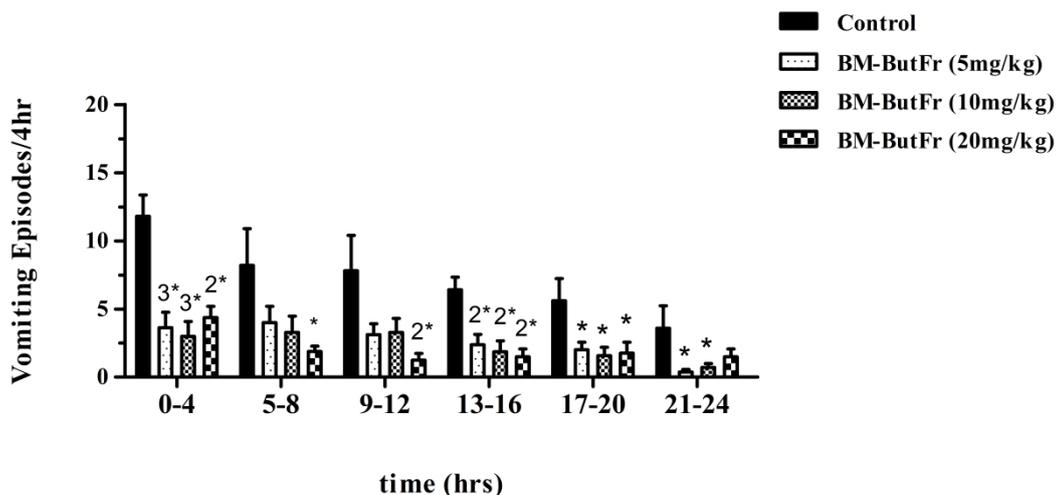
A. Vomiting suppression time profile of metoclopramide (MCP) and N-(2-mercaptpropionyl) glycine (MPG) up to 24 hours.



B. Vomiting suppression time profile of *Bacopa monniera* methanolic fraction (BM-MetFr up to 24 hours).



C. Vomiting suppression time profile of *Bacopa monniera* n-butanolic fraction (BM-But FR) up to 24 hours.



**Figure 4.** Vomiting suppression time profile in pigeons (A) standard metoclopramide (MCP; 30 mg/kg) and N-(2-Mercaptoprionyl) glycine (MPG; 10 mg/kg) (B) *Bacopa monniera* methanolic fraction (BM-MetFr; 10, 20 and 40 mg/kg) and (C) n-butanolic fraction (BM-ButFr; 5, 10 and 20 mg/kg) against cisplatin-induced vomiting during a 24 hr observation period; each bar represents the mean  $\pm$  s.e.m of vomiting episodes occurring during 4 hr periods (n = 7 - 8). Values significantly different from cisplatin control are denoted as \* $p$  < 0.05, <sup>2\*</sup> $p$  < 0.01 <sup>3\*</sup> $p$  < 0.001 (ANOVA followed by Tukey post hoc test).

**Table 1** Effect of BM-MetFr, BM-ButFr, MCP and MPG on cisplatin induced vomiting and jerking during a 24 hr observation period in pigeons.

Drug Treatment	Dose & route	Pigeons n/ vomited	R + V Mean ± sem	Latency (min) Mean ± sem	Jerks Mean ± sem	Wt loss (%) Mean ± sem
Saline + Cisplatin	02ml/kg i.m. + 07mg/kg i.v	6/6	47±5.8	74±6.3	647±162	15.3±1.4
MCP + Cisplatin	30mg/kg i.m. + 07mg/kg i.v	8/8	24±1.3**	204±61.3	351±21	12.3±1.4
MPG + Cisplatin	10mg/kg i.m. + 07mg/kg i.v	8/8	11±5.6***	421±163	225±109*	4.7±1.9**
Saline + Cisplatin	02ml/kg i.m. + 7mg/kg iv	6/6	41±5.2	70±6.9	614±115	12±2
BM-MetFr + Cisplatin	10mg/kg i.m. + 7mg/kg iv	7/7	27±5.6	270±136	610±161	9.1±1.7
	20mg/kg i.m. + 7mg/kg iv	7/7	18±4.4*	156±42	405±167	10.2±1.6
	40mg/kg i.m. + 7mg/kg iv	8/8	13±2.9*	264±132	268±108	8.9±1.7
Saline + Cisplatin	02ml/kg i.m. + 7mg/kg iv	5/5	43±5.8	59±5.3	509±67	16.8±2
BM-ButFr + Cisplatin	5mg/kg i.m. + 7mg/kg iv	8/8	15±3.2***	152±35	309±83	8.3±1.6*
	10mg/kg i.m. + 7mg/kg iv	7/6	13±3.8***	142±46	326±137	5.2±1***
	20mg/kg i.m. + 7mg/kg iv	8/8	12±2.2***	126±14.9	185±38	5.6±1.6***

\* $P < 0.05$ , \*\* $P < 0.01$  \*\*\* $P < 0.001$  as compared to cisplatin control (ANOVA followed by Tukey post hoc test).

Control cisplatin treated animals lost ~ 14.7 % of their starting body weight while, animals treated with BM-ButFr (5, 10 and 20 mg/kg) as well as MPG (10 mg/kg) lost less than 9 % of their starting body weight. These differences compared to the cisplatin control group were found to be statistically significant ( $P < 0.05 - 0.001$ , Table 1). In the control cisplatin treated animals, there were ~ 590 jerking episodes during the 24 hr observation period. Neither fraction of *BM* at those doses studied evoked any effect on any jerking episodes although MPG did significantly reduce cisplatin induced jerks ( $P < 0.05$ , Table. 2).

As shown in table 2, neither BM-MetFr (10, 20 and 40 mg/kg) nor BM-ButFr (5.0, 10 and 20 mg/kg) significantly modified basal levels of the neurotransmitters (NA, DA and 5-HT) or

their metabolites (DOPAC, HVA and 5-HIAA) in the area postrema and brain stem or the small intestine. However, both BM-MetFr (10, 20 and 40 mg/kg) and BM-ButFr (10 mg/kg) significantly decreased ( $P < 0.01$ ) the level of 5-HIAA in the brain stem. Moreover, intestinal NA showed an upsurge ( $P < 0.001$ ) in response to BM-ButFr (20 mg/kg) (Table 2).

**Table 2** Effect of metoclopramide (MCP), *BM* methanolic fraction (BM-MetFr) or *n*-butanolic fraction (BM-ButFr) on basal level of neurotransmitters (ng/mg tissue wet weight) and their metabolites in brain areas and the small intestine of pigeons:

Treatment	NA	DOPAC	DA	5HIAA	HVA	5HT
<b>Area postrema</b>						
Saline	0.610±0.014	0.382±0.111	0.590±0.146	0.158±0.036	0.913±0.095	0.062±0.034
MCP 30mg	0.023±0.005	0.017±0.006	0.025±0.012	0.005±0.001*	0.121±0.063*	0.023±0.001
BM-MetFr 10mg	0.058±0.017	0.047±0.010	0.070±0.007	0.017±0.002	0.346±0.047	0.014±0.002
BM-MetFr 20mg	0.106±0.039	0.168±0.074	0.186±0.066	0.050±0.025	0.238±0.117	0.054±0.034
BM-MetFr 40mg	0.336±0.174	0.092±0.025	0.216±0.089	0.032±0.011	0.476±0.151	0.035±0.014
BM-ButFr 05mg	0.261±0.031	0.043±0.010	0.044±0.105	0.207±0.041	0.426±0.072	0.146±0.050
BM-ButFr 10mg	0.044±0.023	0.115±0.034	0.277±0.054	0.047±0.006	0.356±0.098	0.020±0.004
BM-ButFr 20mg	0.909±0.165	0.313±0.087	0.802±0.210	0.066±0.028	0.854±0.440	0.105±0.057
<b>Brain stem</b>						
Saline	0.094±0.022	0.060±0.020	0.175±0.078	0.060±0.021	0.060±0.016	0.010±0.003
MCP 30mg	0.119±0.033	0.027±0.006	0.044±0.012	0.007±0.001***	0.066±0.031	0.019±0.002
BM-MetFr 10mg	0.040±0.020	0.017±0.006	0.063±0.027	0.011±0.003**	0.051±0.025	0.037±0.019
BM-MetFr 20mg	0.147±0.091	0.052±0.036	0.058±0.040	0.005±0.001**	0.037±0.021	0.019±0.005
BM-MetFr 40mg	0.020±0.009	0.035±0.002	0.022±0.018	0.003±0.001***	0.022±0.015	0.010±0.002
BM-ButFr 05mg	0.108±0.010	0.015±0.001	0.698±0.407	0.067±0.014	0.021±0.003	0.167±0.014***
BM-ButFr 10mg	0.054±0.016	0.021±0.006	0.046±0.011	0.011±0.001**	0.032±0.022	0.011±0.002
BM-ButFr 20mg	0.156±0.097	0.084±0.050	0.178±0.110	0.031±0.001	0.243±0.077***	0.041±0.005
<b>Intestine</b>						
Saline	0.194±0.059	0.067±0.020	0.090±0.064	0.076±0.058	0.056±0.025	0.049±0.016
MCP 30mg	0.138±0.039	0.054±0.025	0.059±0.018	0.097±0.022	0.198±0.102	0.062±0.013
BM-MetFr 10mg	0.117±0.047	0.106±0.047	0.089±0.045	0.061±0.016	0.069±0.032	0.236±0.103
BM-MetFr 20mg	0.114±0.040	0.110±0.053	0.329±0.125	0.077±0.014	0.032±0.012	0.158±0.022

BM-MetFr 40mg	0.015±0.006	0.026±0.003	0.011±0.005	0.028±0.004	0.013±0.009	0.044±0.007
BM-ButFr 05mg	0.204±0.033	0.005±0.001	0.123±0.052	0.268±0.068**	0.077±0.025	0.848±0.187***
BM-ButFr 10mg	0.290±0.083	0.144±0.109	0.289±0.196	0.121±0.028	0.247±0.107	0.106±0.026
BM-ButFr 20mg	1.328±0.271***	0.090±0.044	0.244±0.162	0.051±0.014	0.142±0.052	0.145±0.036

\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  as compared to cisplatin control (ANOVA followed by Tukey post hoc analysis;  $n = 6 - 8$ ).

Cisplatin treatment significantly increased ( $P < 0.001$ ) the concentration of 5-hydroxy tryptamine (5-HT) in the brain stem and small intestine as compared to basal control levels, while no significant increase was observed in the area postrema (Table 3). Treatment with MCP (30 mg/kg) did not modify the concentrations of NA, DOPAC, DA, 5-HIAA and HVA in either brain area or the intestine, but it did significantly decrease ( $P < 0.001$ ) the concentration of 5-HT in the brain stem and intestine as compared to the cisplatin control (Table 3). In addition to its inhibitory effects on 5-HT, MCP also decreased the 5-HIAA concentration in the area postrema (Table 4), while no significant change was observed in the brain stem or small intestine (Tables 3 and 4).

BM-MetFr (10, 20 and 40 mg/kg) and BM-ButFr (5.0, 10 and 20 mg/kg) treatments reduced the concentration of 5HT in the brain stem ( $P < 0.001$ ) and intestine ( $P < 0.001$ , Table 3) as compared to cisplatin control, without any significant effects on NA, DOPAC, HVA and 5HIAA. Furthermore, no significant alteration was observed in the area postrema.

**Table 3** Effect of standard metoclopramide (MCP), *BM* methanolic fraction (BM-MetFr) or n-butanolic fraction (BM-ButFr) on neurotransmitters (ng/mg tissue wet weight) and their metabolites in brain areas and small intestine 3 hours after cisplatin treatment in pigeons:

Treatment	NA	DOPAC	DA	5HIAA	HVA	5HT
<b>Area postrema</b>						
Saline	0.571±0.072	0.389±0.108	0.543±0.130	0.290±0.059	1.374±0.485	0.011±0.001
Cisplatin	1.411±1.160	0.299±0.132	0.073±0.023	0.211±0.079	3.859±3.373	0.316±0.093
MCP 30mg	0.116±0.078	0.106±0.040	0.223±0.103	0.024±0.005	0.069±0.045	0.023±0.005
BM-MetFr 10mg	0.537±0.144	0.129±0.032	0.245±0.066	0.035±0.010	1.301±0.515	0.020±0.009
BM-MetFr 20mg	0.682±0.297	0.358±0.222	0.844±0.585	0.109±0.061	0.447±0.215	0.042±0.009
BM-MetFr 40mg	0.250±0.082	0.206±0.009	0.699±0.143	0.035±0.007	0.393±0.092	0.027±0.006
BM-ButFr 05mg	0.960±0.146	0.492±0.088	0.558±0.128	0.238±0.039	1.603±0.341	1.990±1.646
BM-ButFr 10mg	0.445±0.098	0.110±0.031	0.197±0.058	0.038±0.017	0.319±0.217	0.025±0.011
BM-ButFr 20mg	0.109±0.032	0.058±0.012	0.122±0.020	0.025±0.006	1.317±0.414	0.020±0.003
<b>Brain stem</b>						
Saline	0.071±0.004	0.073±0.012	0.069±0.023	0.017±0.015	0.022±0.010	0.031±0.002
Cisplatin	0.080±0.019	0.130±0.098	0.032±0.001	0.049±0.013	0.016±0.008	0.138±0.018###
MCP 30mg	0.044±0.016	0.015±0.005	0.018±0.011	0.041±0.001	0.042±0.002	0.021±0.001***
BM-MetFr 10mg	0.152±0.050	0.053±0.021	0.011±0.025	0.016±0.004	0.102±0.028	0.012±0.004***
BM-MetFr 20mg	0.145±0.071	0.057±0.021	0.093±0.030	0.025±0.005	0.220±0.062	0.007±0.001***
BM-MetFr 40mg	0.132±0.007	0.097±0.006	0.797±0.086	0.025±0.003	0.222±0.128	0.018±0.001***
BM-ButFr 05mg	0.037±0.010	0.013±0.001	0.010±0.001	0.021±0.004	0.024±0.001	0.090±0.020*
BM-ButFr 10mg	0.068±0.011	0.139±0.126	0.052±0.040	0.055±0.001	0.215±0.077	0.017±0.002***
BM-ButFr 20mg	0.037±0.004	0.083±0.014	0.076±0.016	0.018±0.001	0.289±0.023	0.006±0.001***
<b>Intestine</b>						
Saline	0.374±0.184	0.105±0.040	0.137±0.054	0.410±0.269	0.054±0.022	0.044±0.016
Cisplatin	0.208±0.032	0.015±0.002	0.002±0.000	0.285±0.020	0.035±0.003	0.821±0.137###
MCP 30mg	0.119±0.044	0.060±0.059	0.164±0.127	0.033±0.005	0.086±0.030	0.045±0.006***
BM-MetFr 10mg	0.152±0.050	0.053±0.021	0.116±0.025	0.016±0.004	0.102±0.028	0.012±0.004***
BM-MetFr 20mg	0.229±0.116	0.048±0.031	0.066±0.044	0.028±0.004	0.348±0.185	0.009±0.001***
BM-MetFr 40mg	0.235±0.066	0.101±0.036	0.530±0.235	0.082±0.010	0.290±0.112	0.048±0.021***
BM-ButFr 05mg	0.051±0.024	0.016±0.007	0.040±0.023	0.208±0.026	0.040±0.026	0.420±0.069***
BM-ButFr 10mg	0.087±0.026	0.024±0.013	0.041±0.026	0.021±0.008	0.498±0.353	0.005±0.002***
BM-ButFr 20mg	0.238±0.036	0.105±0.066	0.112±0.035	0.023±0.004	0.784±0.291	0.027±0.008***

Neurotransmitter or metabolite levels significantly different from cisplatin control are denoted by \* $P < 0.05$ , \*\*\* $P < 0.001$ , while Values significantly different as from basal levels are denoted by ### $P < 0.001$  (ANOVA followed by Tukey post hoc analysis;  $n = 6 - 8$ ).

Cisplatin increased the level of DA highly significantly ( $P < 0.001$ ) in the area postrema, while no significant increase was observed in the brain stem or small intestine (Table 4 5). Concentration levels of 5-HT were also raised in the brain stem ( $P < 0.05$ ) and small intestine ( $P < 0.001$ ), without affecting NA, DOPAC, 5-HIAA or HVA levels in the brain stem and intestine or 5-HT in the area postrema (Table 4). Treatment with standard metoclopramide (MCP; 30 mg/kg) significantly decreased the upsurge of DA in the area postrema ( $P < 0.001$ ) and brain stem ( $P < 0.01$ ; Table 4).

Both fractions of *BM* i.e. methanolic (BM-MetFr; 10, 20 and 40 mg) and butanolic (BM-ButFr; 5, 10 and 20 mg) were found to be highly effective in reducing the DA concentration ( $P < 0.001$ ) in the area postrema with respect to the cisplatin control. A similar effect was also seen in the brain stem but with variable statistical significance ( $P < 0.05-0.001$ ; Table 4 5). However, BM-MetFr (20 and 40 mg/kg) failed to attenuate DA concentrations in the brain stem (Table 4). Moreover, in the small intestine, there was a significant reduction ( $P < 0.001$ ) in the levels of 5-HT in response to the *BM* methanolic and butanolic fractions to all the doses tested, with the exception of BM-MetFr (40 mg/kg) and BM-ButFr (5.0 mg/kg). Furthermore, BM-MetFr (40 mg/kg) and BM-ButFr (20 mg/kg) significantly ( $P < 0.05$ ) decreased the level of 5-HIAA in the area postrema (Table 4).

**Table 4** Effect of standard metoclopramide (MCP), *BM* methanolic fraction (BM-MetFr) or *n*-butanolic fraction (BM-ButFr) on neurotransmitters (ng/mg tissue wet weight) and their metabolites in brain areas and the small intestine at 18 hours after cisplatin treatment in pigeons:

Treatment	NA	DOPAC	DA	5HIAA	HVA	5HT
<b>Area postrema</b>						
Saline	0.599±0.084	0.458±0.160	0.535±0.168	0.347±0.084	1.763±0.743	0.012±0.001
Cisplatin	0.247±0.059	0.022±0.008	13.43±4.528###	0.164±0.042	0.395±0.104	0.147±0.044
MCP 30mg	0.161±0.070	0.052±0.023	0.048±0.024***	0.021±0.002*	0.276±0.157	0.010±0.003
BM-MetFr 10mg	0.493±0.166	0.150±0.019	0.365±0.061***	0.059±0.004	1.164±0.272	0.057±0.014
BM-MetFr 20mg	0.467±0.208	0.193±0.058	0.350±0.063***	0.053±0.010	0.576±0.202	0.046±0.009
BM-MetFr 40mg	0.903±0.170	0.075±0.018	0.155±0.033***	0.030±0.004*	0.198±0.045	0.025±0.007
BM-ButFr 05mg	0.808±0.104	0.765±0.152	0.746±0.254***	0.223±0.045	1.177±0.248	0.750±0.243
BM-ButFr 10mg	0.233±0.055	0.039±0.031	0.062±0.044***	0.074±0.032	1.056±0.403	0.037±0.015
BM-ButFr 20mg	0.215±0.036	0.068±0.023	0.146±0.026***	0.024±0.006*	0.901±0.255	0.018±0.003
<b>Brain stem</b>						
Saline	0.069±0.005	0.086±0.017	0.094±0.033	0.018±0.021	0.025±0.017	0.011±0.006
Cisplatin	0.068±0.003	0.021±0.001	0.258±0.057	0.036±0.003	0.019±0.003	0.121±0.008#
MCP 30mg	0.101±0.094	0.001±0.001	0.013±0.013**	0.014±0.004	0.082±0.052	0.014±0.002
BM-MetFr 10mg	0.074±0.066	0.054±0.050	0.021±0.019**	0.035±0.006	0.371±0.161	0.030±0.005
BM-MetFr 20mg	0.241±0.146	0.073±0.033	0.135±0.126	0.024±0.008	0.168±0.051	0.033±0.008
BM-MetFr 40mg	0.384±0.139	0.064±0.015	0.109±0.040	0.015±0.006	0.243±0.100	0.110±0.096
BM-ButFr 05mg	0.072±0.002	0.030±0.002	0.007±0.002***	0.034±0.002	0.010±0.001	0.141±0.005
BM-ButFr 10mg	0.054±0.024	0.025±0.025	0.021±0.016**	0.018±0.003	0.390±0.101	0.007±0.001*
BM-ButFr 20mg	0.128±0.033	0.147±0.030	0.080±0.035*	0.025±0.003	0.216±0.090	0.025±0.003
<b>Intestine</b>						
Saline	0.288±0.237	0.060±0.015	0.058±0.021	0.656±0.403	0.059±0.031	0.045±0.026
Cisplatin	0.228±0.027	0.005±0.001	0.397±0.173	0.390±0.044	0.053±0.006	0.588±0.163###
MCP 30mg	0.177±0.078	0.011±0.003	0.020±0.010	0.022±0.005	0.433±0.384	0.030±0.006***
BM-MetFr 10mg	0.405±0.129	0.281±0.130	0.064±0.330	0.039±0.019	1.112±0.685	0.058±0.024***
BM-MetFr 20mg	0.336±0.144	0.309±0.146	0.122±0.051	0.114±0.023	0.095±0.031	0.188±0.035**
BM-MetFr 40mg	0.545±0.254	0.220±0.104	0.159±0.067	0.113±0.034	0.111±0.043	0.317±0.072
BM-ButFr 05mg	0.152±0.027	0.075±0.034	0.069±0.029	0.227±0.028	0.221±0.131	0.641±0.067
BM-ButFr 10mg	0.103±0.039	0.031±0.031	0.090±0.067	0.066±0.028	1.737±0.360	0.017±0.004***
BM-ButFr 20mg	0.294±0.068	0.235±0.073	0.333±0.161	0.126±0.010	0.594±0.301	0.140±0.024***

Neurotransmitter and metabolites values significantly different from cisplatin control are denoted by \* $P < 0.05$  \*\* $P < 0.01$  \*\*\* $P < 0.001$ , while values significantly different from basal level are indicated by # $P < 0.05$  ### $P < 0.001$  (ANOVA followed by Tukey post hoc analysis;  $n = 6 - 8$ ).

### **Discussion:**

The pigeon is a species that has been used in emesis research for many years and it responds to a number of different emetic stimuli including cardiac glycosides [45,46] ), reserpine [47], sigma receptor ligands [48], and the chemotherapeutic drugs cyclophosphamide [49] and cisplatin [50,51]. In terms of its translational value, it can be used to assay the antiemetic activity of several classes of drug including 5-HT<sub>3</sub> and NK<sub>1</sub> receptor antagonists [16,52] and the glucocorticoids [53]. Investigators have studied lower doses of cisplatin (4.0 mg/kg), where the emetic response can continue for several days and is mediated by vagal and reserpine-sensitive monoaminergic systems [16,54]. In our studies, we used cisplatin at a dose of 7.0 mg/kg and found a reproducible emetic response in all animals for 24 hours without lethality. We also observed that cisplatin at the lower dose of 5.0 mg/kg induced vomiting in 60 % of the animals and this slightly higher dose response relationship with respect to previous studies may be attributed to species differences, environmental factors and food. There is no mechanistically distinct acute or delayed phase of chemotherapy induced vomiting in the pigeon, even though previous studies have monitored emesis for up to 72 hours [16,53]. In our studies, we observed the animals for 24 hours to comply with the ethical use of animals.

Our study is the first to report the antiemetic activity of *BM* in the pigeon model and this indicates that the antiemetic activity should be evaluated in other animal models such as the dog [55] and ferret [56] where delayed emesis can be observed. The major finding of

the present study was that both methanolic and n-butanolic extracts of *BM* possessed antiemetic activity with an efficacy and duration of action superior (MCP). MCP, a D2 and 5-HT<sub>3</sub> receptor antagonist with 5-HT<sub>4</sub> receptor agonist properties, was chosen as the reference standard drug [57]. The dose of MCP that we selected was higher than that required to antagonize cisplatin-induced emesis in other species [58], and was based on a previous study in the pigeon showing activity against reserpine induced emesis [59].

The anti-emetic activity of BM-MetFr prompted the screening of the bacoside rich n-butanolic fraction (BM-ButFr), which proved to be more potent than BM-MetFr indicating a potential inhibitory role of bacosides against vomiting induced by cisplatin. Bacosides have been reported to exhibit anticancer effects [60], potent antioxidant activity [37], and neuroprotective properties against aluminum induced oxidative stress [38,39]. Furthermore, there is a close mechanistic resemblance between the oxidative stress induced by aluminium and cisplatin in the cellular cytoplasm and mitochondria [38,41]. In the long term, *BM* can restore the normal antioxidant defense mechanism of the body [61], which may be useful in protection against delayed emesis. It is possible that this action could further contribute to its antiemetic mechanism of action, since the antioxidant MPG is effective against cisplatin induced vomiting in the pigeon and this finding has also been reported in other species [10,13]. Nevertheless, the efficacious and potent antiemetic nature of BM-ButFr, compared to MCP, may be putatively attributed to the bacoside A components namely bacoside A<sub>3</sub>, bacoside II and bacosaponin C which are detectable in the plant extract (n-butanolic fraction) in concentrations up to 57.91 ± 3.2 µg/ mg, 40.60 ± 0.9 µg/ mg, and 17.23 ± 1.7 µg/ mg, respectively. Likewise the three major components of bacoside A at a total concentration of 115.74 ± 3.9 µg/ mg were identified in BM-ButFr or 38.37 ± 0.7 µg/ gm of the dry powder, which concurs with an earlier report [44]. The presence of steroidal saponins, including bacoside A, which along

with bacopaside 1 constituted more than 96%w/w of the total saponins of *B. monniera* [62], are able to cross the blood brain barrier and modulate enzyme activities, proteins, and neurotransmitters in different brain regions [63-67].

Treatment with *BM* extracts decreased the concentration of 5-HT in the brain stem and intestine (Table 3) three hours following cisplatin administration. The *BM* herb itself has been proved to be antioxidant [68] and this is one of the probable mechanisms by which *BM*-MetFr and *BM*-ButFr reduced the intestinal and brain stem concentration of 5-HT in comparison with MCP (Table 3). Thus, the decrease in 5-HT concentration may arise via protection of enterochromaffin (EC) cells from cisplatin-induced oxidative damage at the intestinal level, and this is an important consideration because 95 % of 5-HT is present in the EC cells of the gastrointestinal mucosa [31]. The decreases in 5-HT induced by both *BM*-MetFr and *BM*-ButFr in the brain stem and intestine are highly relevant to antiemetic propensity. In this context, EC cell 5-HT release can subsequently stimulate 5-HT<sub>3</sub> receptors on vagal afferent fibres triggering an emetogenic 5-HT increase in the area postrema [69] and conversely, a 5-HT level decrement would be antiemetic.

Furthermore, there is evidence of a comparatively high density of 5-HT<sub>3</sub> receptors in the NTS [70,71] and DMV [72] which would support the contention that *BM* administration would be antiemetic.

*BM* treatment had no effect on the levels of neurotransmitters (NA, DA and 5-HT) or their metabolites (DOPAC, HVA and 5-HIAA) in the area postrema during acute time (3 hour after cisplatin administration). However, the area postrema is thought to be involved in the delayed phase of vomiting because ablation of this medullary region suppresses it [73]. Furthermore, in the brain vomiting circuitry, dopamine receptors also located in the area postrema are regarded as the source of both morphine and apomorphine emesis [74-

76]. Conversely, 5-HT is reported to be the primary mediator of the acute cisplatin vomiting response via the NTS [56,77] so it is possible that *BM* influences this acute phase.

Literature regarding the delayed phase of cisplatin induced vomiting is indicative of overlapping mechanisms involving the role of substance P and DA [78,79]. *BM* has been shown to inhibit hyperactivity and dopamine receptor supersensitivity induced by morphine as well as apomorphine climbing behavior in rats [36,80] and comparable findings have also been reported by our laboratory [81]. In the present study, cisplatin treatment resulted in a significant increase ( $p < 0.001$ ) in DA concentration 18 h post administration in the area postrema and a statistically nonsignificant effect in the concentration of DA in the intestine (Table 4). The magnitude of DA upsurge at the level of the area postrema was far greater than in the intestine, as the area postrema is considered to be involved in delayed sickness [73] and is the source of vomiting induction by morphine and apomorphine via the dopaminergic system [74]. Tanihata et al. [54] also observed a slight increase in DA concentration induced by cisplatin (4 mg/kg) in the small intestine when compared to other treatments in pigeons. In another study on minks, Qian and others [82] demonstrated a statistically significant increase ( $p < 0.05$ ) in DA concentration in the area postrema and ileum after cisplatin treatment (7.5 mg/kg). In a study in the least shrew animal model, cisplatin (10 mg/kg) increased ( $p < 0.05$ ) the DA concentration during both phases of vomiting while duodenal DA levels were unaffected [83]. In contrast, *BM*-MetFr and *BM*-ButFr treatments decreased the dopamine upsurge in the area postrema and brain stem (Table 4). These findings provide evidence in favor of an antiemetic effectiveness of *BM* extracts for prolonged protection against cisplatin emetogenesis which is contingent upon DA function. Such antidopaminergic activity of

*BM* extracts is entirely complementary to previously reported neurochemical studies from this laboratory [43]. Furthermore, the *BM*- induced decrease in 5-HIAA and 5-HT levels in the area postrema and intestine 18 h after cisplatin treatment may well be the manifestation of additional mechanism(s) involved in the anti-emetic properties of the herbal extracts.

Both *BM* extracts failed to alter basal levels of neurotransmitters or their metabolites in the brain stem with the exception of a fall in 5-HIAA. Consequently, it might be argued that such comparative selectivity of their neurochemical activity for the area postrema and small intestine is an indicator of prospective safety and tolerability. Apart from a possible application in emetogenic chemotherapy, *BM* has other additional properties including antinociceptive activity [84,85] and efficacy in acute stress or chronic unpredictable stress [64] both of which may be advantageous in patients undergoing cancer chemotherapy [86].

In conclusion, the present study has disclosed that *BM* extracts provide a long lasting suppression of vomiting induced by cisplatin, whereby anti-serotonergic and anti-dopaminergic activities occur over early and late emetic phases in a continuum. Thus during the early phase, anti-serotonergic effects predominate while in the later phase there is an anti-dopaminergic aftermath. *BM*-BuFr, which is rich in bacoside A, showed superior antiemetic activity, thus implicating the role of bacoside A in the antiemetic activity of *BM*, though further studies are required to investigate the involvement of its individual components. Moreover, *BM* is known to be non-addictive, and has a tolerable safety profile making it worthy of further investigation as an anti-emetic agent, or at least an adjunct, against cisplatin induced vomiting in other animal models as well as in man.

**Materials and Methods:****Animals:**

Pigeons of either sex (mixed breed, Department of Pharmacy, University of Peshawar, Pakistan) weighing between 250 - 350g were used. They were housed in groups of eight at 22 - 26 °C under a 12h:12h light/dark cycle and had free access to food (locally available food; millet + wheat) and water before and during experimentation. All the experimental procedures were approved by the Ethical Committee, Department of Pharmacy, University of Peshawar (Ref. No 5/pharm) and are in accordance with the UK animal scientific procedure Act, 1986.

**Drugs and chemicals:**

Cisplatin ( $\geq 99.9\%$ ) (Korea United Pharm. Inc. Korea), metoclopramide (MCP; GlaxoSmithKline Pakistan Ltd.), N-(2-Mercaptopropionyl) glycine (MPG  $\geq 98\%$ ; Sigma Aldrich Germany), NA  $\geq 98\%$ , DOPAC  $\geq 98\%$ , DA  $\geq 99\%$ , 5HIAA  $\geq 98\%$ , HVA  $\geq 98\%$ , 5-HT  $\geq 99\%$  (Acros Organics, Belgium), HPLC grade acetonitrile (99.9%), methanol (99.9%) and 1-octane sulphonic acid sodium salt ( $>98\%$ ) (Fisher scientific U.K), sodium dihydrogen orthophosphate sodium (99%) and EDTA ( $\geq 99\%$ ) (Merck), Bacoside A<sub>3</sub> ( $\geq 95\%$ ), Bacoside II ( $\geq 99\%$ ) and Bacosaponin C ( $\geq 90\%$ ) (a gift from Prof. Dr. Ikhlas Khan, the National Center for Natural Products Research, University of Mississippi USA), commercial grade methanol, n-hexane, n-butanol and acetone (Haq Chemicals Peshawar).

**Extraction of *Bacopa monniera* and fractionation:**

The plant was collected in November from Rumalee stream near Quaid-e-Azam University, Islamabad Pakistan. The plant was authenticated by Prof. Dr. Muhammad Ibrar, Department of Botany, University of Peshawar and a specimen was deposited in the herbarium (Voucher

No 7421). The aerial parts were separated, shade dried and coarsely powdered. A 1 kg sample of the resultant plant material was extracted with *n*-hexane (6L), and then with acetone (5L) to remove fats and chlorophyll-type pigments. The product was then extracted using commercial grade methanol (1.5L) in a soxhlet apparatus to yield 28 g. The resultant material was subfractionated to obtain the *n*-butanol fraction (yield 1.6 grams), which is reported to be rich in bacosides [87]. The methanolic fraction (BM-MetFr) and the bacoside rich *n*-butanolic fraction (BM-ButFr) of the plant were dissolved in distilled water prior to antiemetic testing.

#### **Gross phytochemical investigation:**

*BM* was screened preliminarily, for the presence of glycosides [88] namely triterpenoids [89], tannins, flavonoids, saponins [90] and alkaloids [89,91].

#### **Quantification of Bacosides in *BM* methanol and *n*-butanol fractions:**

High performance liquid chromatography coupled with UV was used for the quantification of Bacoside “A” major components bacoside A<sub>3</sub>, bacoside II and bacosaponin C using our own method with slight modification [44]. Briefly, 5mg of BM-MetFr or BM-ButFr was dissolved in 5mL of HPLC grade methanol, centrifuged at 3000 rpm for 10 minutes and filtered through 0.45μ filter. 300μL of the filtered solution was further diluted with methanol to make up a volume of 5mL. HPLC system equipped with LC-20AT double pumps (Shimadzu, Japan), a rheodyne injector of 20μL loop, SPD-20A UV detector (PDA) and puorspher C18 column (250mm × 4.6mm × 4μm particle size) was used. The mobile phase consisted of 0.2% phosphoric acid and acetonitrile (62:38, v/v). The mobile phase PH was adjusted to 3 using 3M NaOH.

**Drug formulation:**

Cisplatin was dissolved in normal saline by heating up to 60°C, cooled to 40 - 45°C and immediately administered. The methanolic and n-butanolic fractions of *BM* were dissolved in normal saline by gentle agitation and sonicated to get a clear solution for *in vivo* administration.

**Drug administration:**

Cotton wool and methylated spirits were used to sterilize the skin prior to drug administration. Intravenous and intramuscular administrations were done through the brachial wing vein and chest muscle, respectively using Neoject 2ml non-pyrogenic syringes with sharp painless needles (27G × 1/2" for the i.v. route, and 23G × 1" for the i.m. route). Immediately, after the last injection, the animals were put back in the specially designed confining/observation cages and the incident counts of R + V and latency to first vomit were recorded for 24 h. At the end of experiment, body weight loss was calculated. Subsequently, the animals were decapitated to terminate the experiment.

**Antiemetic assay:**

On the day of the experiment, the pigeons were placed in individual cages specially designed for video observation. A preliminary study was conducted to evaluate the optimal dose of cisplatin to induce vomiting. Thus, cisplatin (2.0 – 10mg/kg) was administered intravenously via the brachial wing vein at 0 minutes (t = 0) [54]. The behavior of each pigeon was then recorded for 24 h. Food and water were available during the observation period and each animal was used only once. The response with or without oral expulsion was considered as one vomiting episode [92]. The latency to first vomit and the number of vomiting episodes were recorded. A vomiting episode was considered to be completed when the pigeon adopted relaxed posture. Jerking episodes, which are indicative of vomiting intensity, were

also recorded. In subsequent antiemetic studies, cisplatin was used at 7.0 mg/kg, i.v. to elicit an emetic response to enable assessment of the potential antiemetic action of the *BM* fractions, MCP and MPG. In these studies, BM-MetFr, BM-ButFr, MCP and MPG or respective vehicles, were administered 30 minutes before cisplatin administration.

#### **Tissue sampling for neurotransmitters analysis:**

Two discrete parts of the brain (brain stem and area postrema) as well as the intestinal samples 5 – 6 cm from the pylorus were used for the neurotransmitter analyses and the effects of BM-MetFr, BM-ButFr and MCP were investigated. At the end of each experiment, animals were decapitated and the brain areas and intestinal samples were rapidly dissected and placed on an ice cold plate (0°C). The dissection of brain parts was carried out according to the atlas of Karten and Hodos [93] and Henri M. Duvernoy [94]. After decapitation of experimental animals, the dorsal surface of the skull was exposed by making an incision along the midline, and the temporal muscles were stripped off to expose the skull bone. After exposing the skull, bones and meninges were carefully removed in such a way to expose the brain hemispheres, especially to make the brain stem prominent from the ventral aspect. The long strip of capillaries stretching from the obex on the median line to the lateral angles of the fourth ventricle (area postrema) was dissected followed by dissection of the brain stem. Jejunal samples of about 2 cm were rapidly removed and washed with ice cold saline. The collected samples were rapidly frozen on an ice plate and stored at -80°C until analysis.

#### **Determination of neurotransmitters and their metabolites:**

Tissue samples were homogenized in cold 0.2 % perchloric acid (PCA) at 5000 rpm with the help of Teflon glass homogenizer (Wise stir HS 30 E). After centrifugation (Centurion UK) at 12000 g/min (4°C) the samples were filtered through a 0.45 micron filter. Neurotransmitters and their metabolites were analyzed using a high performance liquid

chromatography system (HPLC, Shimadzu, Japan) coupled with electrochemical detection (ECD, ESA Coulochem III model 5300), a pump (model LC-20AT), and an analytical column (Teknokroma 3 x 150, 3 $\mu$ m). The mobile phase consisted of 94 mM sodium dihydrogen orthophosphate, 40 mM Citric acid, 2.3 mM sodium 1-octane sulphonic acid, 50  $\mu$ M EDTA, and 10 % acetonitrile (pH 3). The flow rate was maintained at 0.6 mL/min. The standards used were NA hydrochloride, DOPAC, DA hydrochloride, 5HIAA, HVA and 5-HT. The HPLC method was reproducible and all the neurotransmitters and their metabolites were separated within 13 minutes.

### **Statistical analysis:**

The differences between means were evaluated using a one way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests.  $P < 0.05$  was considered as statistically significant. The animals which showed complete suppression of R + V were not included in statistical analyses for latency. Data represent the mean  $\pm$  SEM unless otherwise indicated.

### **Supporting information**

The structures of the major bioactive components of bacoside A and details of the phytochemical screening of BM-MetFr are available as Supporting Information

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## Conflict of Interest

The authors have no conflicts of interest to declare.

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