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Phytochemical profiling and evaluation of emetic potential of *Chrozophora Tinctoria* whole plant extracts

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Chrozophora tinctoria commonly known as turnsole, giradol or dyer·s croton belongs to the family Euphorbiaceae. The plant is traditionally used as an emetic, to cure abdominal pain, menstrual problems, ulcers of the mouth, jaundice, migraine, cathartic, laxative and to treat warts and expel the worms. In the present study, we used Gas Chromatography-Mass Spectrometry (GC-MS) to detect the chemical constituents of the methanolic extract (MECT) and n-butanol fraction (NBFCT)of *C. tinctoria*. The MECT and NBFCT were tested for antioxidant activity by DPPH radical scavenging assay, acetylcholinesterase (AChE) inhibitory potential and acute toxicity. Both the samples were also subjected to emetic activity against antiemetic agents i.e. dimenhydrinate and metoclopramide. The compounds tentatively identified in GC-MS in both samples were mostly fatty acids. About eleven compounds in MECT and seven in NBFCT were detected. These included biologically active compounds like imipramine which has antidepressant activity, undecane has enzyme inhibitory and carcinogenic properties, butylated hydroxytoluene has antioxidant activity, pentadecanoic acid, 14-methyl-, methyl ester has antimicrobial activity, hexadecanoic acid, methyl ester has nematicide, pesticide, hypocholesterolemic property. Similarly, 9-Octadecenoic acid, methyl ester, (E)- has antiandrogenic, cancer preventive and dermatitigenic activity, 1,2-Benzenedicarboxylic acid diisooctyl ester has cytotoxic potential, 1-Monolinoleoylglycerol trimethylsilyl ether has anti-arthritic, diuretic and antiasthma properties while Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl has been used as an antiseptic, skin conditioning agent and emollient. MECT showed significant antioxidant potential with IC_{50}= 31µg/mL as compared to NBFCT with IC_{50}=67µg/mL. Both the samples significantly inhibited the acetylcholinesterase. The highest percent inhibition was observed at 1000 µg/mL for MECT and NBFCT which were 83.33±3.51% and 73.33±3.06% with IC_{50} equal to 40µg/mL and 110µg/mL respectively. Similarly, MECT and NBFCT were found toxic at 1g/kg concentration and showed a dose-dependent manner. Both the samples exhibited emesis and diarrhea at 1g/kg and were highly toxic at 4g and 5g/kg by inducing tremors, drooping of wings, lethargy and up to 75% mortality in pigeons. The emetic potential produced at 1g, 2g and 3g/kg by MECT and NBFCT was checked against antiemetic drugs dimenhydrinate (2mg/kg, I.M) and metoclopramide (2mg/kg, I.M). It was observed that emesis induced by MECT and NBFCT was decreased by both the antiemetic drugs, but metoclopramide (2mg/kg, I.M) was more effective than dimenhydrinate (2mg/kg, I.M). The current study scientifically validates the folkloric use of *Chrozophora tinctoria* as an emetic agent. Further research work is required to screen the studied plant for isolated compounds and their emetic potential.

Keywords: GC-MS; antioxidant; acetyl cholinesterase inhibitor; emetic; acute toxicity

INTRODUCTION

Emesis, also known as vomiting, is the unpleasant effect in which material present in the stomach is expelled out through the mouth. This expulsion out of stomach material is called vomiting, while the commotion of vomiting is called nausea. Emesis occurs when a person intakes some chemical like copper sulphate, water with maximum salt, zinc sulphate and apomorphine, etc. When someone ingests toxic substances then emetic agents are used to expel those toxic substances (Mughal and Mahboob, 2013). Vomiting is controlled by the vomiting Center present in

the medulla oblongata of the brain, the Chemoreceptor Trigger Zone (CTZ) also known as Area Postrema (AP) is present on the floor of the 4th ventricle of the brain. This CTZ is activated by various neurotransmitters like acetylcholine, norepinephrine, serotonin, histamine, Leu-enkephalin and Met-enkephalin, etc which trigger the vomiting. Similarly, toxins, dopamine and nicotine also provoke vomiting. The CTZ that signals the vomiting center to start vomiting includes the gastrointestinal tract, vestibular system and the higher Center in the thalamus and cortex. Overeating, blotted stomach, morning sickness and motion sickness, etc. are some common problems that lead to vomiting without the interference of CTZ (Silbernagl et al. 2009). Emesis is common in patients of cancer who face chemotherapy for the treatment of cancer. Emesis also occurs due to, drug side effects, old history of motion sickness, gastro paresis and in the first trimester of pregnancy. Many anti-emetic drugs are used to target the emetic receptor sites but these drugs also have various side effects like hypertension, sedation, hallucinations, drying of mouth and dysphoria (Ahmed et al. 2013). Plant-natural products have been used by mankind for health purposes before the advancement in medicine. Traditional medicine available today passed on from the ancient, is still practiced by human beings. Since an early age, plants have been an ideal source of medicine. Due to the low cost and minimum side effects on human health, phytomedicine become the center of attraction for many scientists around the globe for many years. And therefore medicinal plants are regarded as a storehouse of many bioactive chemicals having many therapeutic properties. A large scale of therapeutic activities is relatedto medicinal plants such as anti-microbial, anti-nociceptive, Anti-inflammatory, Anti-viral, Anti-Tumor and anti-malarial, etc. (Aye et al.2019). An antioxidant may be a natural or synthetic compound that functions to slow down lipid oxidation when present at a low concentration compared to an oxidizable lipid. Antioxidants work by scavenging free radicals such as butylated hydroxytoluene, tocopherols and some plant bioactive compounds that inhibit lipid oxidation by reducing peroxyl and alkoxyl radicals to stable compounds (Alamed et al. 2009). Many studies carried out on medicinal plants proved that phytochemicals have an active role as antioxidants and anticancer (Al-Rimawi et al. 2022). It has been reported that several medicinal plants are used as antiemetic drugs which were tested against various emetic inducers in different animal models. It has been discussed that many herbal remedies used as emetic/antiemetic have not undergone scientific investigations (Ahmed et al. 2014).

C. tinctoria is a member of the family Euphorbiaceae. The plant is called “turnsole” “giradol or dyer's croton” as the plant produces a blue-purple colorant “turnsole”. The colour obtained from the turnsole is used as a colouring agent in many foodstuffs. Therefore, the plant is well-known for producing dye substances and flavonoids. The plant is annual, erect and about 1.5-2.5 feet in height. The flowering season is from June to September. The plant is found in Asia, Africa and Europe. In Pakistan and India, the plant is found in temperate and subtropical regions. The plant is rich in alkaloids, coumarins, diterpenoids, flavonoids, glycosides and xanthones. The plant has antimicrobial, antipyretic and anti-nociceptive potential (Sher et al. 2018). It also has antioxidant, antifungal and cytotoxic, antidiabetic potential and to treat hair loss, as the plant is rich in secondary metabolites (Snafi, 2015). Flavonoids isolated from the plant fractions exhibited the antiosteoporosis influence (Naim et al. 2018). Traditionally the genus *Chrozophora* is used to cure pain (abdominal pain, joint pain), fever, menstrual problems, ulcers of the mouth, skin burns, jaundice, and migraine and to expel worms. *Chrozophora tinctoria* is also reported as an emetic, cathartic, and to treat warts (Ahmed et al. 2014). The phytochemical studies of plants showed that the plant contains alkaloids,flavonoids, glycosides, coumarins, diterpenoids and xanthones (Sher et al. 2018). It has also been reported that all the species of the genus *Chrozophora*exhibited the presence of flavonoids (Tabussum et al. 2013) and about 35 flavonoids were detected in various species of Genus *Chrozophora* (Marzouk et al. 2016). Similarly, glucose, sucrose, fructose, raffinose, ribose and arabinose were detected in *Chrozophora* through HPLC analysis (Ahmed, 2003) likewise, five flavonoid glycosides were identified from aerial parts of *Chrozophora tinctoria* by the HPLC method, which was apigenin 7-O-b-d-[6-(3,4dihydroxybenzoyl)]-glucopyranoside (*Chrozophorine*), quercetin 3-O-rutinoside, apigenin 7-O-b-d-[(6-pcoumaroyl)]-glucopyranoside, acacetin 7-O-rutinoside and apigenin 7-O- b-d-glucopyranoside (Delazar et al. 2006). In the current study, the methanolic extract of *Chrozophora tinctoria* (MECT) and the n-butanol fraction of *Chrozophora tinctoria* (NBFCT) were tested for bioactive compound by Gas chromatography-mass spectrometry, antioxidant activity, acetylcholinesterase inhibition, acute toxicity and it possible emetic potential in model animals.

MATERIALS AND METHODS

Chemicals and solvents

The analytical grade chemicals used in the present study were copper sulphate, Methanol, Ethyle acetate, dichloromethane, n-butanol, n-hexane, acetyl cholinesterase and normal saline, Similarly, the drugs used were dimenhydrinate galantamine and metoclopramide (Maxolon ®),

Instruments

The instruments used were an electric grinder, Filter Papers (Whatman's filter paper), vacuum rotary evaporator, glass funnel, China dishes, Petri dishes, water bath, separating funnel, analytical balance, flasks, magnetic stirrer, spectrophotometer, feeding tube, large and small cages.

Plant collection and identification

Fully mature plants of *Chrozophora tinctoria* were collected in August and September from the district Mohmand (34.22^0 N to 71.48^0 E), Khyber Pakhtunkhwa, Pakistan. The plant was identified in the flora of Pakistan (Taxon I.D 220002849) and also by Dr. Sher Wali (Ph.D.), Assistant Professor, Department of Botany, Islamia College Peshawar. A voucher number Icp-2021/49 representing the *C. tinctoria* was kept in the Herbarium of the botany department, Islamia College Peshawar. The plants were then cleaned and dust was removed by washing with tap water. The plants were then shade-dried at room temperature. The shade-dried plants were cut into small pieces and were then powdered with the help of an electric grinder.

Preparation of crude extraction

About 5kg of powdered plant material was macerated in methanol with random shaking for three weeks. The mixture was then filtered with filter paper. All the filtered methanolic solution was concentrated by a rotary evaporator to get the crude methanolic extract. After obtaining the crude methanolic extract, it was dried in a water bath at 45^0C. The solidified extract was then divided into two portions. One portion (first portion) was used as methanolic extract while the other portion (second portion) was fractioned with different solvents (Bakht et al. 2011).

Fractionation

Crude methanolic extract (second portion) was dissolved in distilled water. After adding a considerable amount of distilled water and making a paste of methanolic extract, the mixture of water and the methanolic extract was transferred to the separating funnel. First of all, the required amount of n-hexane was mixed to the mixture in a separating funnel. The separating funnel was shaken softly and fixed in a stand so that two layers (upper and lower) of n-hexane and aqueous appeared in the separating funnel. The upper n-hexane fraction was collected in a flask and the lower aqueous layer was fractioned twice with fresh n-hexane. All the n-hexane fractions were gathered and dried using a rotary evaporator. The dried n-hexane fraction was obtained in a China dish. After this, the same procedure was carried out with an increase in polarity for dichloromethane, and then ethyl acetate and n-butanol correspondingly, this process of fractionation is called Solvent-Solvent fractionation. The methanolic extract of *C. tinctoria* (MECT)obtained at the first stage (first portion) and the n-butanol fraction of *Chrozophora tinctoria* (NBFCT) obtained at the last stage was used for further work (Sher et al. 2017).

Gas Chromatography-Mass Spectrometry (GC-MS)

The GC-MS of MECT and NBFCT was performed to identify the phytochemical compounds were present in them. The samples were examined by GC-MS Model QP 2010 plus (Shimadzu) fitted with an autosampler (AOC-20S) and an auto-injector (AOC-20i). Pure Helium gas (99.99%) with a flow rate of 1ml/min was used as a carrier gas. The chromatographic segregations were executed on a capillary column (TR-5MS) with the following specifications: a length of 30 meters, an internal diameter of 0.25mm and a thick film of 0.25µm. Dichloromethane was used as a reagent. The injection temperature, ion source temperature (EI) and interface temperature were all 250^0C with 100 KPa Pressure and 70eV ionizing energy. The temperature of the oven was set from 60˚C (hold for 2 min) to 240˚C. Both MECT and NBFCT were diluted in a relevant solvent and were filtered with Whatman's filter paper. Each sample of volume 1µL was then injected into the injector using a syringe with a split ratio of 1:50. The temperature of the column started at 50^0C for a minute and then with a flow rate of 15^0C/min the temperature was raised to 150^0C. After this at a rate of 2.5^0C/min, the temperature was elevated to 175^0C and kept for 5 minutes. Then, the temperature was changed to 220^0C with an interval of 3.5^0C/min and controlled for 3 minutes. Full-scan mass spectra were performed from 40–550m/z. The characterization and identification of phytochemicals were based on the retention time of gas chromatography. The phytochemical compounds in both samples were identified by matching the mass spectra collected with those of standard spectra from the GC-MS library (Alam et al. 2014).

Pharmacological activities:

Animals: Healthy and mature pigeons of either sex, weighing 250g-350g were selected. The conditions under which the pigeons were considered healthy are, pigeons having no runny eyes, non-lethargic, no nasal dropping no weight loss, normal stool, regular feeding and usual flying.

Invitro-experiments

DPPH radical scavenging assay

Free radicle scavenging ability of MECT and NBFCT was calculated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) following the procedure of [Kamal *et al.* 2015]. The activity started by making the solution of DPPH (24g) in 100mL of methanol. A stock solution (1mg/mL) of MECT and NBFCT was prepared in methanol and then different concentrations i.e. 125, 250, 500 and

1000µg were obtained. Then 0.1mL of these different concentrations of both samples were mixed with 3mL of DPPH solution in methanol. Then the solution was kept in the dark and incubated at 23^0C for 30 minutes. After 30 minutes, the absorbance was measured at 517nm using a UV spectrophotometer. Ascorbic acid was used as positive control. Then Percent scavenging activity was calculated as; $[(A_0 - A_1)/A_0] \times 100$. Where A_0 is the control absorbance and A_1 is the sample absorbance (Kamal et al. 2015).

Acetylcholinesterase Assay:

The MECT and NBFCT were tested to inhibit the acetyl cholinesterase. Both the samples were tested with increasing concentrations (125, 250, 500, and 1000 µg/mL) using a spectrophotometer following Ellman's method. In this method the enzymes hydrolysis the substrate acetylthiocholine resulting in the product thiocholine. This thiocholine reacts with DTNB (Ellman's reagent) which produces two products i.e. 2-nitrobenzoate-5-mercaptothiocholine and 5-thio-2nitrobenzoate detected at 412 nm by Spectrophotometer. Concentrations of Galantamine (Positive control) were also prepared in the same manner as MECT and NBFCT. The solutions of tested samples and the positive control were incubated at 37◦C for about 20 minutes. The absorbance was measured at 412 nm. The inhibition of the enzyme by tested samples and control was measured from the absorption rate with a change in time (Ellman et al. 1996; Ayaz et al. 2014)

The percent inhibition was calculated as,

Enzyme inhibition (%) = 100−percent enzyme activity; Percent enzyme activity (%) = 100×V/Vmax.

Where (V_{max}) is an enzyme activity in the absence of an inhibitor.

***In Vivo Experiments*:**

Acute Toxicity

The acute toxicity test of MECT and NBFCT was accomplished on pigeons following the available protocols of (Joshi et al. 2007) with some minute modifications. First of all the animals were divided into two groups (n=8). 1st group (Negative control) was served with distilled water (6 mL/kg, P.O) while the 2nd group received the tested samples. Each sample was given orally at a concentration of 0.1, 0.3, 0.5, 1, 2, 3, 4, and 5g/kg as a single dose. All the doses were given orally with the help of a feeding tube and toxicity symptoms like stopping feeding and drinking, vomiting, diarrhea, tremors, drooping of wings, lethargy and mortality were noticed for 72 hours (Joshi et al. 2007).

Emetic Activity:

The emetic potential of MECT and NBFCT was carried out on pigeons according to the protocols of (Hu et al. 2003; Ono et al. 2017) with some modifications. The animals were divided into 7 groups (n=8). Groups 1st, 2nd, and 3rd were served distilled water and 1g, 2g and 3g/kg doses of the tested samples. Group 4th declared as negative control received the distilled water (6mL/kg) only, while group 5th, 6th, and 7th were given the tested samples (MECT and NBFCT) at a concentration of 1g, 2g and 3g/kg, Antiemetic drug dimenhydrinate was injected (2mg/kg, I.M) 30 minutes before the administration of distilled water to 4th group and tested samples to 5th, 6th, 7th group. Immediately after sample receiving, the parameters like First jerk time, first vomit time, the number of jerks, the number of vomits and the weight of vomit were noted. the response of pigeons with expulsion was called vomiting and without expulsion were called jerks. The emetic potential of MECT and NBFCT was also checked against the antiemetic drug Metoclopramide. The same activity was performed for metoclopramide (2mg/kg, I.M) replacing dimenhydrinate ((Hu et al. 2003; Ono et al. 2017).

RESULTS

GC-MS:

GC-MS of MECT:

About eleven different phyto active compounds were tentatively detected in MECT with a similarity index (S.I) greater than 500 as shown in Table 1. The peaks presented in the chromatogram were matched with a database of known components mentioned in the GC-MS library. The GC-MS analysis of MECT showed the presence of many biologically active compounds (Table 2) at various retention times. These compounds were mostly fatty acids and hydrocarbons.

GC-MS of NBFCT

Similarly, seven phytoactive compounds from the NBFCT were tentatively detected with a similarity index of more than 500, after matching the retention times presented by the NBFCT chromatogram with the GC-MS library as shown in Table 3. These seven biologically active compounds (Table 4) were mostly hydrocarbons, fatty acids and benzene-containing derivatives.

Pharmacological activities

***In-vitro* experiments**

DPPH radical scavenging assay:

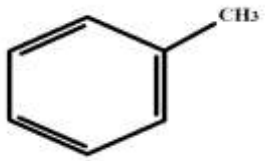
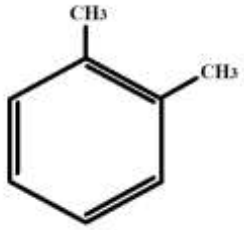
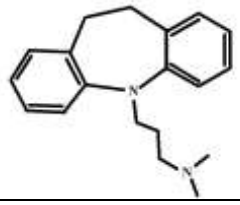
DPPH radical scavenging activity of MECT and NBFCT is given in Table 5. The MECT and NBFCT showed significant percent inhibition of DPPH in dose-dependent manner. Lower IC_{50} values suggest higher free radical scavenging activity. The highest free radical scavenging activity was obtained from MECT with the lowest IC_{50} value of 31µg/mL followed by NBFCT which is 67µg/mL, when compared with standard Ascorbic acid

Values were expressed as mean ± SEM. Statistical significance was determined using IC_{50} valuesthroughBiostatasoftware. IC_{50}= Half-maximal inhibitory concentration; DPPH =2,2-diphenyl-1-picrylhydrazyl.

Table 1: Phytochemical compounds detected in MECT through GC-MS

S.No.	R.Tim (Min)	Name of Compound	Simi. Ind.	Prob.	Peak area %	M.F	M.W	Lib
1	1.21	Toluene	828	25.72	0.23	C_7H_8	92	replib
2	1.75	o-Xylene	943	41.03	19.37	C_8H_{10}	106	replib
	3.25	Imipramine	641	36.57	0.02	$C_{19}H_{24}N_2$	280	nist_msms
4	3.36	Undecane	606	23.31	0.15	$C_{11}H_{24}$	156	replib
5	6.16	Butylated Hydroxytoluene	787	48.08	0.04	$C_{15}H_{24}O$	220	replib
6	6.42	Pentadecanoic acid, 14-methyl-, methyl ester	834	58.97	0.18	$C_{17}H_{34}O_2$	270	MAINLIB
7	12.42	Hexadecanoic acid, methyl ester	897	73.48	0.45	$C_{17}H_{34}O_2$	270	replib
8	13.50	10-Octadecenoic acid, methyl ester	646	6.67	0.35	$C_{19}H_{36}O_2$	296	MAINLIB
9	27.64	1-Monolinoleoylglycero l trimethylsilyl ether	674	41.20	0.01	$C_{27}H_{54}O_4Si_2$	498	MAINLIB
10	37.42	Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl	622	10.63	0.01	$C_{12}H_{38}O_5Si_6$	430	MAINLIB
11	39.68	Octasiloxane, 1,1,3,3,5,5,9,9 ,11,11,15,15-h	650	25.60	0.01	$C_{16}H_{50}O_7Si_8$	578	MAINLIB

Table 2: Reported biological activities of the identified photoactive compounds of MECT

S.No	Compound name	Compound structure	Biological Activity	References
1.	Toluene		It has Psychoactive effects when inhaled intentionally. used in paints, plastic production, lacquers, thinners, glues,	[25]
2.	o-Xylene		It is known for its wide usage in tissue processing, staining and coverslipping in the histology laboratory. Exposure to o-xylene causes symptoms of nausea, vomiting and gastric discomfort	[26]
3.	Imipramine		Anti-depression.	[27]
4.	undecane		antimicrobial, enzyme inhibitor, carcinogen, it is used as a mild sex attractant in cockroaches and moths.	[28]

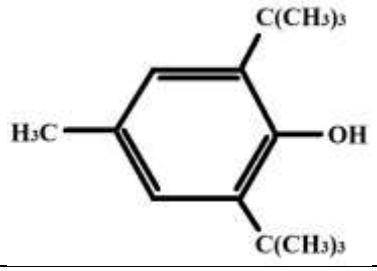
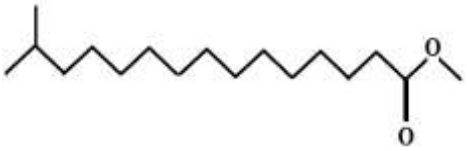
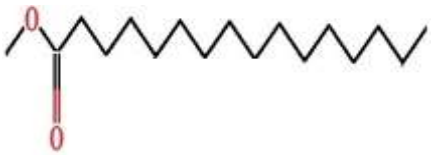
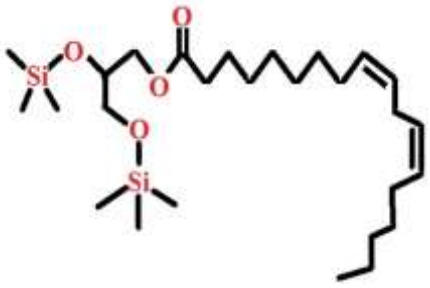
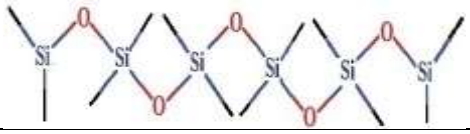
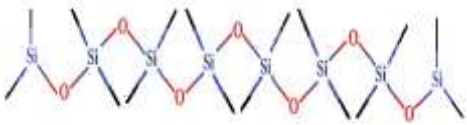
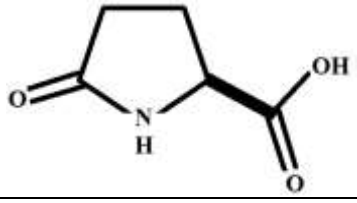
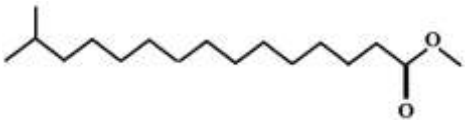
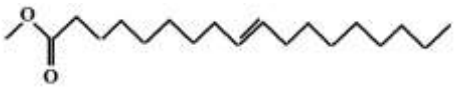
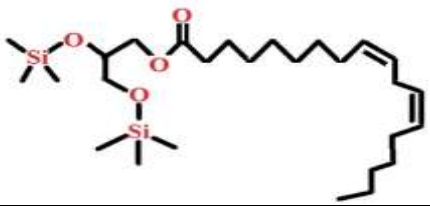
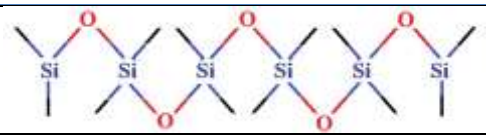
				
5.	Butylated Hydroxytoluene		foods preservative and antioxidant	[29]
6.	Pentadecanoic acid, 14-methyl-, methyl ester		Antioxidant, Antimicrobial,	[30]
7	Hexadecanoic acid, methyl ester		Nematicide, Antioxidant, Pesticide, Hypocholesterolemic,Hemolytic,5-Alpha reductase inhibitor	[31]
8	10-Octadecenoic acid, methyl ester		Antimicrobial, Antioxidant, Hypocholesterolemic	[32]
9	1-Monolinoleoylglycerol trimethylsilyl ether		Antimicrobial, Antioxidant, Anti-inflammatory, Antiarthritic, Diuretic, Antiasthma,	[33]
10	Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11, 11-dodecamethyl		Antimicrobial, Antiseptic, Skin-Conditioning Agent-Emollient,	[34]
11	Octasiloxane,1,1,3,3,5 ,5,9,9,11,11,15,15-h		antimicrobial	[35]

Table 3: Phytochemical compounds identified in NBFCT

S.No	R.Tim (Min)	Name of Compound	Simi. Ind.	Prob.	Peak area %	M.F	M.W	Lib
1	5.44	Pidolic Acid	666	12.91	0.01	$C_5H_7NO_3$	129	MAINLIB
2	12.19	Pentadecanoic acid, 14-methyl-, methyl ester	770	67.68	0.02	$C_{17}H_{34}O_2$	270	MAINLIB
3	16.60	9-Octadecenoic acid, methyl ester, (E)-	721	8.72	0.01	$C_{19}H_{36}O_2$	296	Replib
4	27.64	1,2-Benzenedicarboxylic acid diisooctyl ester	521	50.24	0.02	$C_{24}H_{38}O_4$	390	Replib
5	36.95	1-Monolinoleoylglycerol trimethylsilyl ether	560	46.55	0.01	$C_{27}H_{54}O_4Si_2$	498	MAINLIB
6	41.38	Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11 -dodecamethyl	538	21.95	0.01	$C_{12}H_{38}O_5Si_6$	430	MAINLIB
7	41.96	Octasiloxane, 1,1,3,3,5,5,9,9, 11,11,15,15-h	545	30.29	0.01	$C_{16}H_{50}O_7Si_8$	578	MAINLIB

Table 4: Reported biological activities of the identified photoactive compounds of NBFCT

S.No	Compound name	Compound Structure	Biological Activity	References
1	Pidolic Acid		It is useful to combat productivity losses due to water scarcity, enhances the rate of photosynthesis and antioxidant defenses, and maintains osmotic and water balance	[36]
2	Pentadecanoic acid, 14-methyl-, methyl ester		Antibacterial, antifungal and antioxidant	[30]
3	9-Octadecenoic acid, methyl ester, (E)-		Anti-inflammatory, antiandrogenic cancer preventive, hypocholesterolemic, 5-alpha reductase inhibitor, insectifuge	[32]
4	1,2-Benzenedicarboxylic acid diisooctyl ester		Cytotoxic, antifungal	[37],[38]
5	1-Monolinoleoylglycerol trimethylsilyl ether		Antioxidant Antimicrobial Antiarthritic Antiinflammatory Diuretic, Antiasthma,	[33]
6	Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-		Antiseptic, Antimicrobial, Skin-Conditioning Agent-Emollient,	[34]

	dodecamethyl			
7	Octasiloxane,1,1,3,3,5,5,9,9 ,11,11,15,15-h		Antibacterial Antifungal,	[35]

Acetylcholinesterase Inhibitory Assay:

MECT and NBFCT

Both the MECT and NBFCT exhibited acetylcholinesterase inhibitory potential at various concentrations as shown in Table 5. Both the samples were tested at 125, 250, 500 and 1000µg/mL concentrations and showed significant AchE inhibition at the highest concentration when compared to standard galantamine (IC_{50}=05µg/mL). It was observed that MECT exhibited the highest percentage of AChE inhibition i.e. 83.33±3.51% at the highest concentration i.e.1000µg/mL with IC_{50} of 40µg/mL. Similarly, NBFCT also showed 73.33±3.06% of AchE inhibition at 1000 µg/mL with IC_{50} of 110µg/mL.

Acute Toxicity:

MECT and NBFCT

The parameters of acute toxicity of MECT and NBFCT are presented in Table 4. It was observed that doses from 0.1g to 0.5g were found safe, the acute toxicity started from 1g/kg in the form of vomiting and diarrhea in both tested samples. Both MECT and NBFCT were highly toxic at 4g/kg and 5g/kg concentrations by inducing tremors, drooping of wings, lethargy and mortality. Similarly, at 4 and 5g/kg concentrations, the MECT exhibited 50% mortality while at the same doses, 50% and 75% mortality was observed for NBFCT.

Emetic Activity:

Dimenhydrinate Model:

The emetic potential showed by MECT and NBFCT at 1g, 2g and 3g/kg were checked against dimenhydrinate as shown in Tables 5 and 6. It was observed that the emetic potential of MECT was attenuated by dimenhydrinate (2mg/kg, I.M) by increasing the 1st jerk and 1st vomit time and decreasing the number of jerks and vomits when compared with D.W + MECT and control group as shown in Table 7.

Values were expressed as mean ± SEM; one-way ANOVA followed by Dunnett's test was applied using GraphPad prism software. (* p≤0.05; ** p≤0.01; *** p≤0.001); NO. Number; Min, Minutes; DIM, dimenhydrinate; DW, distilled water; PO, per orally/by orally; IM, Intramuscularly, MECT, methanolic extract of *C. tinctoria*.

Similarly, the emetic activity of NBFCT was also reduced to some extent by dimenhydrinate (2mg/kg, I.M) when compared with D.W and NBFCT and the control group as shown in Table 8.

Table 5: Percent inhibition of DPPH by MECT and NBFCT.

Compound name/ Plant name	Samples	Concentration (µg/mL)	% Inhibition of DPPH	DPPH IC_{50} (µg/mL)
Ascorbic acid	Standard	1000	88.33±3.51	19
		500	83.67±3.06	
		250	78.00±2.00	
		125	67.33±3.00	
Chrozophora tinctoria	MECT	1000	80.33±3.51	31
		500	78.00±2.00	
		250	69.00±4.00	
		125	62.33±2.52	
	NBFCT	1000	71.67±2.08	67
		500	67.00±2.00	
		250	63.67±3.51	
		125	55.33±4.51	

Values were expressed as mean ± SEM. Statistical significance was determined using IC_{50} values through Biostata software. IC_{50}= Half-maximal inhibitory concentration; DPPH =2,2-diphenyl-1-picrylhydrazyl.

Metoclopramide Model:

The emetic effect induced by MECT and NBFCT at 1g, 2g and 3g/kg was also tested against metoclopramide (2mg/kg, I.M) as shown in Tables 7 and 8. It was observed that the emetic potential of MECT was significantly ($p < 0.001$) decreased by metoclopramide (2mg/kg, I.M), as the 1st jerk time and 1st vomit time was increased and the number of jerks and number of vomits were decreased by metoclopramide as compared to D.W + MECT and control group as shown in Table 9.

A similar pattern of emetic activity was observed for NBFCT against metoclopramide as shown in Table 10. Which introduced that metoclopramide significantly ($p< 0.001$) reduced the emetic effect of NBFCT. The time for the 1st jerk and vomit was increased and the number of jerks and vomits was decreased when compared with D.W + NBFCT and the control group.

Table 6: Percent inhibition of acetylcholinesterase by MECT and NBFCT

Compound name/ Plant name	Samples	Concentration (µg/mL)	% AchE inhibition	IC_{50} (µg/mL)
Galantamine	Standard	1000	95.67±2.52	05
		500	87.33±2.52	
		250	82.67±3.06	
		125	77.00±3.00	
Chrozophora tinctoria	MECT	1000	83.33±3.51	40
		500	73.00±3.00	
		250	68.33±4.51	
		125	61.00±2.00	
	NBFCT	1000	73.33±3.06	110
		500	65.00±2.65	
		250	57.67±2.08	
		125	50.33±2.52	

Values were expressed as mean ± SEM. Statistical significance was determined using IC_{50} values through Biostata software. IC_{50}= Half-maximal inhibitory concentration; AChE = Acetylcholinesterase;

Table 7: Acute toxicity of MECT and NBFCT in pigeons.

Samples	Doses (g).(mL) /kg	Feeding /drinking	Emesis	Diarrhoea	Tremors	Drooping of wings	Lethargy	Mortality (%)
			Number of vomits	Number of Wet ferces				
Distilled water	6	-	0.00±0.00	0.00±0.00	-	-	-	0.00±0.00
MECT	0.1	-	0.00±0.00	0.00±0.00	-	-	-	0.00±0.00
	0.3	-	0.00±0.00	0.00±0.00	-	-	-	0.00±0.00
	0.5	-	0.00±0.00	0.00±0.00	-	-	-	0.00±0.00
	1	-	4.00±1.00	9.33±2.52	-	-	-	0.00±0.00
	2	-	5.00±2.00	13.33±2.52	-	-	-	0.00±0.00
	3	-	7.00±2.00	14.00±4.58	-	-	Less	0.00±0.00
	4	Less	7.67±2.52	14.33±4.51	less	Observed	More	50.00±0.00
	5	Stopped	9.00±2.65	16.33±2.52	More	Observed	More	50.00±0.00
NBFCT	0.1	-	0.00±0.00	0.00±0.00	-	-	-	0.00±0.00
	0.3	-	0.00±0.00	0.00±0.00	-	-	-	0.00±0.00
	0.5	-	0.00±0.00	0.00±0.00	-	-	-	0.00±0.00
	1	-	3.00±1.00	10.33±3.51	-	-	-	0.00±0.00
	2	-	4.67±1.53	14.67±3.06	-	-	Less	0.00±0.00
	3	Less	5.00±1.73	15.00±5.00	less	Observed	More	25.00±0.00
	4	Stopped	6.00±2.00	16.00±3.61	More	Observed	Most	50.00±0.00
	5	Stopped	7.33±3.51	19.33±3.51	More	Observed	Most	75.00±0.00

Values were expressed as mean ± SEM. (-) = Normal

Table 8: The emetic activity of MECT against dimenhydrinate

Treatment	Doses ml/kg g/kg (P.O)	1st Jerk time (min)	1st vomit time (min)	No. of jerks	No. of vomits	Wt. of vomits
D.W + MECT	1	18.67±3.06	19.67±3.51	2.00±1.00	5.33±3.06	4.17±1.48
	2	15.67±2.08	17.67±2.52	3.33±1.53	6.67±2.08	5.17±1.58
	3	12.33±2.52	13.33±3.06	4.33±2.52	8.33±3.06	6.53±2.47
Administration of dimenhydrinate (2mg/kg. I.M.) 30 minutes before giving extract/fractions/ distilled water.						
Dim + D.W (control)	6	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
MECT + DIM	1	26.67±1.53***	31.67±1.53***	1.33±0.58	4.00±1.00*	3.47±0.47**
	2	24.00±2.00***	27.33±2.52***	2.00±0.00*	4.33±0.58*	2.97±0.15*
	3	23.00±2.00***	25.33±1.53	2.33±0.58**	4.67±1.15*	3.20±0.40**

Values were expressed as mean ± SEM; one-way ANOVA followed by Dunnett's test was applied using Graph pad prism software. (* p≤0.05; ** p≤0.01; *** p≤0.001); NO. Number; Min, Minutes; DIM, dimenhydrinate; DW, distilled water; PO, per orally/by orally; IM, Intramuscularly, MECT, methanolic extract of *Chrozophora tinctoria*.

Table 9: The emetic activity of NBFCT against dimenhydrinate

Treatment	Doses ml/kg g/kg (P.O)	1st Jerk time (min)	1st vomit time (min)	No. Of jerks	No. Of vomits	Wt. of vomits
D.W + NBFCT	1	26.33±3.51	29.33±3.51	2.33±0.58	3.33±1.53	2.27±1.01
	2	21.67±4.04	24.00±4.00	2.67±1.15	5.33±2.52	3.53±1.36
	3	19.33±3.51	21.33±4.51	3.33±0.58	6.67±2.08	4.60±1.87
Administration of dimenhydrinate (6mg/kg I.M) 30 minutes before giving extract/fractions/ distilled water.						
Dim + D.W	6	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
NBFCT + Dim	1	39.67±1.53***	41.67±1.53***	1.00±0.00	2.67±1.15	1.10±1.83
	2	33.00±3.00***	36.00±3.00***	1.33±0.58	3.00±1.00	1.90±0.96
	3	31.33±1.53***	34.33±3.06***	1.67±1.15	3.67±1.15	2.37±0.72

Values were expressed as mean ± SEM.; one-way ANOVA followed by Dennett's test was applied using Graphpad prism software. Where p≤0.05 was considered statistically significant. (* p≤0.05; ** p≤0.01; *** p≤0.001); NO. Number; Min, Minutes; DIM, dimenhydrinate; DW, distilled water; PO, per orally/by orally; IM, Intramuscularly, NBFCT, the n-butanol fraction of *Chrozophora tinctoria*.

Table 10: The emetic activity of MECT against metoclopramide

Treatment	Doses ml/kg g/kg (P.O)	1st Jerk time (min)	1st vomit time (min)	No. of jerks	No. of vomits	Wt. Of vomits
D.W + MECT	1	20.00±2.00	21.67±3.51	2.33±0.58	4.67±2.08	3.00±1.35
	2	16.33±1.53	17.00±2.65	2.67±0.58	6.00±1.00	4.17±1.98
	3	11.00±3.00	12.00±3.00	3.33±0.58	7.33±2.08	5.47±2.10
Administration of metoclopramide (6mg/kg I.M.) 30 minutes before giving extract/fractions/ distilled water.						
Meto + D.W (Control)	6	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
MECT + Meto	1	47.33±2.52***	49.00±2.00***	0.67±0.58	1.00±0.00	0.47±0.15
	2	43.67±3.51***	46.33±3.06***	1.33±0.58	1.33±0.58	0.67±0.35
	3	33.00±3.00***	35.33±3.51***	2.00±0.00*	2.00±0.00**	0.93±0.25

Values were expressed as mean ± SEM.; one-way ANOVA followed by Dunnett's test was applied using Graphpad prism software.where p≤0.05 was considered statistically significant. (* p≤0.05; ** p≤0.01; *** p≤0.001); NO. Number; Min, Minutes; METO, Metoclopramide; DW, distilled water; PO, per orally/by orally; IM, Intramuscularly, MECT, methanolic extract of *Chrozophora tinctoria*.

Table 11: The emetic activity of NBFCT against metoclopramide.

Treatment	Doses ml/kg g/kg (P.O)	1st Jerk time (min)	1st vomit time (min)	No. of jerks	No. of vomits	Wt. of vomits
D.W + NBFCT	1	24.00.±2.00	25.33±2.52	1.67±0.58	3.00±1.00	1.60±0.44
	2	19.00±4.00	20.67±4.51	2.00±1.00	4.67±1.53	2.97±1.27
	3	15.67±3.51	16.33±3.51	3.00±1.00	6.33±1.53	4.27±1.85
Administration of metoclopramide (6mg/kg I.M.) 30 minutes before giving extract/fractions/ distilled water.						
Meto +D.W (Control)	6	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
NBFCT + Meto	1	51.00±2.00***	52.33±2.08***	0.67±0.58	1.00±0.00	0.47±0.21
	2	44.67±4.51***	47.00±4.58***	1.33±0.58	1.33±1.15	0.63±0.42
	3	38.33±3.06***	42.00±2.65***	2.00±0.00*	2.00±0.00**	1.23±0.21*

Values were expressed as mean ± SEM; one-way ANOVA followed by Dunnett's test was applied using Graphpad prism software.where p≤0.05 was considered statistically significant. (* p≤0.05; ** p≤0.01; *** p≤0.001); NO, Number; Min, Minutes; METO, Metoclopramide; DW, distilled water; PO, per orally/by orally; IM, Intramuscularly, NBFCT, the n-Butanol fraction of *Chrozophora tinctoria*.

DISCUSSION

Herbal medicines have been appreciated throughout the history of mankind by using them to treat different infectious diseases. Many photochemical achieved from plants have the pharmacological ability to fight against microbes. The pharmacological activities of these herbs are due to the presence of primary and secondary metabolites present in them. It has been reported that about 1200 secondary metabolites have been isolated from these medicinal plants. Plants used these secondary metabolites for their defensive purposes against microorganisms, insects and herbivores. However, these secondary metabolites are valued highly by human beings due to their medicinal properties (Mahire & Patel, 2020) The GC-MS analysis of MECT and NBFCT showed the presence of numerous biologically active compounds as shown in Tables 1, 2 and 3, 4. The compound present in MECT like Toluene has psychoactive effects (Cruz et al. 2014). o-Xylene is known for its wide usage in tissue processing, staining and coverslipping in the histology laboratory[40] Imipramine is used as anti-depression (Ramirez et al. 2016). Undecane has enzyme inhibitor and cisarcinogen (Krishnamoorthy & Subramaniam, 2014). Butylated Hydroxytoluene has antioxidant activity (Likhitrungrat et al. 2010). Hexadecanoic acid, methyl ester is used as a nematicide, pesticide and hypocholesterolemic (Sudha et al. 2013). 10-Octadecenoic acid, methyl ester has antimicrobial activity and decrease blood cholesterol [32] Similarly, compounds present in NBFCT like Pidolic Acid are useful to combat productivity losses due to water scarcity, it enhances the rate of photosynthesis and antioxidant defenses (Jiménez-Arias et al. 2019). Pentadecanoic acid, 14-methyl-, methyl ester has antibacterial and antifungal properties. 1,2-Benzenedicarboxylic acid diisooctyl ester has cytotoxic activity (Krishnan et al. 2014; Rahman & Anwar, 2006) 1-Monolinoleoylglycerol trimethylsilyl ether has antiarthritic, anti-inflammatory, diuretic and antiasthma properties (Parthipan et al. 2015). Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl is used as Antiseptic, Skin-Conditioning agent-emollient[34] and Octasiloxane,1,1,3,3,5,5,9,9,11,11,15,15-h has antimicrobial activity (Senthil et al. 2016).

DPPH radical scavenging test is used to evaluate the antioxidant properties of MECT and NBFCT, it was observed that both the samples showed antioxidant potency. This significant antioxidant activity of MECT might be due to the presence of a maximum number of phytochemicals that were already reported antioxidants such as Pentadecanoic acid, 14-methyl-, methyl ester (Elaiyaraja et al. 2018). Hexadecenoic acid, methyl ester (Sudha., 2013).10-Octadecenoic acid, methyl ester (Belakhdar., 2015) and 1-Monolinoleoylglycerol trimethylsilyl ether (Parthipan et al. 2015) in MECT. And Pentadecanoic acid, 14-methyl-, methyl ester (Elaiyaraja et al. 2018) and 1-Monolinoleoylglyceroltrimethylsilyl ether (Parthipan et al. 2015) in NBFCT. MECT showed significant antioxidant properties as compared to NBFCT.

Acetylcholine (Ach) is a neurotransmitter present abundantly in the body and is responsible for cholinergic transmission. Acetylcholinesterase (Ache) belongs to the family of enzymes and plays a vital role in the termination of nerve impulses at cholinergic synapses by hydrolyzingthe neurotransmitter acetylcholine into choline and acetic acid (Mukherjee et al. 2007). The stopping of Acetylcholinesterase (Ache) is an auspicious strategy against many diseases like,senile dementia, ataxia, Parkinson's disease and Alzheimer's disease, etc.[44]It has been reported that phytochemicals extracted from plants inhabit the acetylcholinesterase (Nath & Yadav, 2015). The chemoreceptor trigger zone (CTZ) located in the brain is activated by various neurotransmitters (Acetylcholine, Serotonin, Dopamine, Histamine Substance "P" and GABA etc.) in the brain that induces vomiting. Acetylcholine (neurotransmitter) mostly stimulates the vomiting reflex through the Vagal afferent pathway (Nath & Yadav, 2015). The amount of

acetylcholine increases with the inhibition of the enzyme acetylcholinesterase.This acetylcholine passesthe message to the CTZ through the vagus efferent pathway which in turn provokes emesis. The MECT significantly inhibited acetylcholinesterase with an IC_{50}=40µg/ml. The highest percent inhibition was shown at 500 µg/ml and 1000 µg/ml concentrations which were 73.00±3.00% and 83.33±3.51% respectively. Similarly, NBFCT also showed 73.33±3.06% of AChE inhibition at 1000 µg/mL with IC_{50}=110µg/mL.

In developing countries, people use traditional herbal medicines for the treatment of numerous diseases. For this purpose, various studies have been carried out to know the pharmacological potential of herbal remedies. However, very little work was done to know the toxicity of such remedies, as several studies have described that many medicinal plants do cause toxicity. And for this purpose, many scientific approaches have been made to know the toxicity of such herbs (Zafar et al. 2018). In the present study, we investigated the acute toxicity of MECT and NBFCT in pigeons. The toxic effect of MECT and NBFCT and its appearance on the general behavioral pattern of pigeons are shown in Table 4. Both the samples were tested safe from 0.1g to 0.5g. and were found toxic at 1g/kg and onward by inducing vomiting and diarrhea in the animals. MECT was highly toxic at 4g/kg and 5g/kg by inducing tremors, drooping of wings, lethargy and mortality upto 50%. Similarly, NBFCT was also highly toxic at 4g/kg and above by inducing all the mentioned parameters along with 75% mortality at 5g/kg.

Emesis is an unpleasant effect and common digestive problem. and many antiemetic drugs control vomiting. In the present study, the emesis induced by MECT and NBFCT at 1g/kg, 2g/kg and 3g/kg were checked against antiemetic drugs Dimenhydrinate (2mg/kg, I.M) and Metoclopramide (2mg/kg, I.M). Dimenhydrinate is antihistamine, antivertigo and Anti-emetic agent. Dimenhydrinate is a histamine receptor H1 antagonist with anticholinergic properties which results in the reduction of vomiting. It also exerts antiemetic effects by interacting with other neurotransmitter systems like dopamine, norepinephrine, serotonin and acetylcholine [48].Similarly, Metoclopramide is an antiemetic drug it acts by inhibiting acetylcholine, blocking dopamine (D2) and serotonin (5-HT3) receptors in the CTZ locatedin the brain and inhibiting emesis (Iqbal et al. 2016). It was observed that dimenhydrinate (2mg/kg, I.M) reduced the emetic potential of MECT by increasing the 1st jerk and 1st vomit time and decreasing the number of jerks and vomits when compared with D.W + MECT and control group as shown in Table 5. Similarly, dimenhydrinate (2mg/kg, I.M) also attenuated the emetic activity of NBFCT when compared with D.W and NBFCT and the control group as shown in Table 6.It was also revealed that Metoclopramide (2mg/kg, I.M) significantly ($p < 0.001$) reduced the emetic potential of MECT, as the drug increased the 1st jerk time and 1st vomit time and decreased the number of jerks and number of vomits as compared to D.W+MECT and control group as shown in Table 7. Similarly, metoclopramide (2mg/kg, I.M) also significantly reduced the emetic activity of NBFCT when the parameters for emetic activity were compared with D. W+NBFCT and the control group as shown in Table 8. It was observed that emesis induced by MECT and NBFCT at 1g/kg, 2g/kg and 3g was decreased by both the antiemetic drugs. However, the antiemetic drug metoclopramide (2mg/kg, I.M) was more effective than the antiemetic drug dimenhydrinate (2mg/kg, I.M) by decreasing the emetic potential of both MECT and NBFCT. It was also concluded from the acetylcholinesterase activity that both the tested samples MECT and NBFCT inhibited the acetylcholinesterase and allowed acetylcholine to accumulate and provoke emesis. However, the antiemetic drug metoclopramide (2mg/kg, I.M) successfully inhibited acetylcholine and decreased the emetic potential of both samples more than dimenhydrinate (2mg/kg, I.M).

CONCLUSIONS

In conclusion, the study effectively validates the traditional use of *Chrozophora tinctoria* as an emetic agent. Through Gas Chromatography-Mass Spectrometry (GC-MS) analysis, the methanolic extract (MECT) and n-butanol fraction (NBFCT) of *C. tinctoria* were found to contain several biologically active compounds, predominantly fatty acids, which possess various medicinal properties. Both MECT and NBFCT demonstrated significant antioxidant activity and acetylcholinesterase inhibitory potential, with MECT showing superior efficacy. Acute toxicity tests indicated dose-dependent toxicity, with both extracts causing notable emetic and diarrheal effects at higher concentrations. The emesis induced by these extracts was effectively mitigated by antiemetic drugs, particularly metoclopramide. These findings corroborate the traditional use of *C. tinctoria* and highlight the necessity for further research to isolate specific compounds and thoroughly evaluate their emetic potential.

Supplementary materials

Not applicable

Author contributions

Conceptualization, A.A.S methodology, A.I.; software, M.A.; validation, F.Z.F.; formal analysis, A.; investigation, A.Q.; resources, S.T.; data curation, M.N.; writing-original draft preparation, S.I.; writing-review and editing, S.I; visualization, M.A; supervision, A.A.S and M.A.; project administration, M.N.; funding acquisition, M.A. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement
The study was approved by the ethical board of Islamia College Peshawar, Pakistan. The ethical approval no. is EC/Bot/ICP-673.

Informed Consent Statement
Not applicable.

Data Availability Statement
The data associated with these findings are available from the corresponding author upon request.

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Conflict of interest
The authors declared that present study was performed in absence of any conflict of interest.

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