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Original Article

GCMS characterization and biological potential of the seeds and aerial part of *Galium tricorne* Stokes

Caracterização GCMS e potencial biológico das sementes e parte aérea de *Galium tricorne* Stokes

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Abstract

Natural products have long been proven very effective against various challenging diseases including cancer and bacterial infections. *Galium tricorne* is one of the important source of natural products, which has not been explored till date in spite of its profound ethnomedicinal prominence. The current study has been designed to explore the biological potential of *G. tricorne* and to extract and isolate chemical constituents from its aerial part and seeds respectively along with identification of their chemical constituents. Phytochemical screening was performed to figure out the presence of secondary metabolite in *G. tricorne*. Crude Methanolic extract (Gt.Crd), which was obtained from the aerial part while the fatty acids were extracted from the seeds, which were later on analyzed by GCMS. Similarly, Well Diffusion and MTT method were used for antibacterial activity and cancer cell line assay respectively. To evaluate the cytotoxic potential, brine shrimps were used. Likewise, in Gas Chromatography-Mass Spectroscopy (GC-MS) analysis a total number of 23 compounds were identified in Gt.Crd extract out of which 7 compounds were sorted out to have some sort of toxicity profile. In the same fashion, 5 fatty acids were identified in the seeds of *G. tricorne*. Moreover, among the fractions, chloroform fraction (Gt.Chf) exhibited greater zone of inhibition (ZOI) 20.37 mm followed by Gt.Crd 18.40 mm against *S. aureus* and *S. pyogenes* respectively. In cytotoxicity Gt.Chf was more active followed by ethyl acetate fraction (Gt.Eta) by exhibiting 88.32±0.62% (LC_{50}=60 µg/mL) and 73.95±2.25% (LC_{50}=80 µg/mL) respectively at 1000 µg/mL concentration of the tested sample. Gt.Chf exhibited greater cell line inhibitory activity (IC_{50}=61 µg/mL) against HeLa cell line. Similarly, Gt.Crd displayed IC_{50} values of 167.84 µg/mL and 175.46 µg/mL against HeLa and NIH/3T3 cell line respectively. Based on the literature review and screenings, it may be concluded that the aerial part and seeds of *G. tricorne* are the rich sources of bioactive compounds. The results of the current study also authenticate the scientific background for the ethnomedicinal uses of *G. tricorne*.

Keywords: *Galium tricorne*, phytochemical, antimicrobial, cytotoxic, MTT assay.

Resumo

Os produtos naturais têm se mostrado muito eficazes contra várias doenças desafiadoras, incluindo câncer e infecções bacterianas. O gálio tricorne é uma importante fonte de produtos naturais, ainda pouco explorada, apesar de sua profunda proeminência etnomedicinal. O presente estudo foi desenvolvido para explorar o potencial biológico de *G. tricorne* e extrair e isolar constituintes químicos de sua parte aérea e sementes, respectivamente, juntamente com a identificação de seus constituintes químicos. A triagem fitoquímica foi realizada para descobrir a presença de metabólito secundário em *G. tricorne*, extrato metanólico bruto (Gt.Crd) que foi obtido da parte aérea enquanto os ácidos graxos foram extraídos das sementes, que posteriormente foram analisadas por GCMS. Da mesma forma, os métodos Well Diffusion e MTT foram usados para atividade antibacteriana e ensaio de linha de células cancerígenas, respectivamente. Para avaliar o potencial citotóxico, foram utilizadas artémias. Da mesma forma, na análise de cromatografia gasosa-espectroscopia de massa (GC-MS) um número total de 23 compostos foi identificado no extrato de Gt.Crd, dos quais 7 compostos foram selecionados para ter algum tipo de perfil de toxicidade. Da mesma forma, 5 ácidos graxos foram identificados nas sementes de *G. tricorne*. Além disso, entre as frações, a fração clorofórmio (Gt. hf) apresentou maior zona de inibição (ZOI), 20,37 mm, seguida de Gt.Crd 18,40 mm contra *S. aureus* e *S. pyogenes*, respectivamente. Na citotoxicidade Gt.Chf foi mais ativo seguido pela fração acetato de etila (Gt.Eta), exibindo 88,32±0,62% (LC50=60 µg/mL) e 73,95±2,25% (LC50=80 µg/mL) respectivamente a 1000 µg/ concentração de mL da amostra testada. Gt.Chf exibiu maior atividade inibitória da linha celular (IC50 = 61 µg/mL) contra a linha celular HeLa. Da mesma forma, Gt.Crd apresentou valores de IC50 de 167,84 µg/mL e 175,46 µg/mL contra linha celular HeLa e NIH/3T3, respectivamente. Com base na revisão de literatura e triagens, pode-se concluir que a parte aérea

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e as sementes de *G. tricorne* são as ricas fontes de compostos bioativos. Os resultados do presente estudo também autenticam a base científica para os usos etnomedicinais de *G. tricorne*.

Palavras-chave: *Galium tricorne*, fitoquímico, antimicrobiano, citotóxico, ensaio MTT.

1. Introduction

From thousands of years plants have been used medicinally as an important source of drugs (Samuelsson, 2004). People of the world especially developing countries have been using medicinal plants for the causal treatment, healing and prevention of illnesses as raw material since ancient times (Stasi, 1996; Toledo et al., 2023) and still used traditionally as protective remedy (Omar et al., 2020). The use of herbal products in Brazil is a common practice wired by the cultural diversity (Pio et al., 2019). Most of the plants in the world flora are unknown because among the 250,000-500,000 plant species, not more than 10% plant species were evaluated for pharmacological, chemical and biological parameters (Verpoorte, 1998). It has been estimated that the Brazilian flora is one of the largest flora comprising of about 120 thousand species and only 1% has been evaluated for their pharmacological and phytochemical properties (Vilar et al., 2008). Crude extract of the medicinal plants are used, which may contain a variety of metabolites with often unknown biological properties (Konan et al., 2007). For this purpose, medicinal plants have to be evaluated for their safety, efficacy and to confirm its traditional uses (Stone, 2008). The natural product obtained from the medicinal plants have been passed through various biological and pharmacological tests which lead to the discovery of new lead compounds of pharmaceutical importance and great possibilities to develop as drugs, pesticides, dyes and fragrances (Hamburger and Hostettmann, 1991; Debiasi et al., 2023). Phytochemicals are present as naturally occurring substances in most of the plants and inclined great economic importance (Enechi et al., 2013; Santana et al., 2022). Phytochemicals like, tannins, terpenoids, phenols, flavonoids, alkaloids and saponins are of great importance (Yadav and Agarwala, 2011). Secondary metabolite saponins possess better antifungal activity (Porsche et al., 2018), tannins with antimicrobial activity (Dong et al., 2018), some of the alkaloids for the treatment of HIV infection and flavonoids having potent anticancer activity (Kurapati et al., 2016). Cancer is a condition characterized by uncontrollable proliferation of cells in any part of the body leads to the destruction of various tissues and organs. The use of the medicinal plants for the treatment of cancer was not new for the indigenous communities in various countries of the world for which documented proof is available (Lee and Houghton, 2005; Sowemimo et al., 2010). It was documented that from 1981 to 2006 about 47% of the 155 clinically approved oncological drugs were derived from natural products either semi synthesized, unmodified compounds or synthesized based on natural product (Newman and Cragg, 2007).

The Rubiaceae family is also known as the madder, bedstraw or coffee family and one of the fourth largest angiosperm family (Xu and Chang, 2017). This family is composed of 650 genera and about 13,000 species (Bremer and Manen, 2000). The plants related to this family are not only distributed in tropical and subtropical regions but also in cold and temperate regions of the northern Canada and Europe (Pereira et al., 2006). This family is classified in three subfamilies and 43 tribes (Bremer and Manen, 2000). In Pakistan Rubiaceae family is represented by 33 genera and 87 species (Nazimuddin and Qaiser, 1989).

To evaluate the safety and possible side effects of medicinal plants, toxicological studies were conducted on plant extract in animals (Menegati et al., 2016). The Galium genus is composed of annual and perennials herb (Edwards et al., 2003). Galium word is derived from the Greek word 'gala' meaning milk. It is also called "bed straw" (Hocking, 1997). Traditionally, Galium species are used to coagulate milk due to the presence of enzyme, therefore, it is called "yogurt herb" (Ergun et al., 1999). Various species of the Galium are used for variety of pathological conditions like, gout, treatment of GIT disorders, choleretic, and epilepsy (Temlzer et al., 1996). Pharmacological activities of the genus Galium have been reported in the literature. Secondary metabolites obtained from Galium species were found to possess cardiovascular, anti-inflammatory and antitumor properties (Hsu et al., 1997; Mitova et al., 2002). It was also observed that Galium species also carry mild diuretic property, which increases the urine volume and flow and reduces stone formation (Gillespie and Stapleton, 2004). Methanolic extract and ethyl acetate fraction of *Galium aparine* have notable cytotoxic and apoptotic activity on human MCF-7 breast cancer and Caco-2 colon cancer cells (Aslantürk et al., 2017). Sub species *Longipedunculatum* of *Galium tricornutum* is also used as a folk medicine for painful conditions, skin infections as well as diuretic in kidney disorders (Shah et al., 2006). This specie has tendency of better antibacterial and antifungal activities (Jan et al., 2009). *Galium tricorne* belongs to family Rubiaceae and one of the unexplored specie of the genus Galium. Through literature, it has been revealed that other species of this family possess different biological activities and various metabolites, have been isolated from other species and Galium genus, but this plant is not being explored yet. The aim of this study was to evaluate the antibacterial, cytotoxicity and cancer cell line toxicity potentials of *Galium tricorne* crude extract (Gt.Crd) and its various fractions. Similarly, for the identification of compounds GC-MS technique was used.

2. Materials and Methods

2.1. Plant collection, extraction and fractionation

The plant was collected in the blooming season of April and May from Village of Ghoriwala named Kot Mehtar located in District Bannu Khyber Pakhtunkhwa (KP), Pakistan. It was identified by Dr. Tahir Iqbal, taxonomist in the Department of Botany, University of Science & Technology Bannu KP Pakistan and deposited in the

Herbarium of the said University with voucher No. SA/2018/Gt/01 for future reference. Large amount of Gt.Crd extract was obtained because of maceration process. For the high yield of the plant sample maceration process is effective (Aspé and Fernández, 2011). Furthermore, separating funnel was used for the fractionation process. For the extraction process, solvents were selected on the basis of polarities. *n*-hexane is a non-polar therefore, non-polar or least polar compounds were extracted first according to the law "like dissolves like". So, the non-polar compounds were extracted readily and the polar compounds were extracted with polar solvents (Snyder, 1974). The Gt.Crd extract and their fractions were subjected to various biological activities and identifications of compounds in Gt.Crd and fatty acid in the seeds of *G. tricorne* were checked out through GC-MS technique.

2.2. Phytochemical screening

For the phytochemical screening Gt.Crd of plant was used for the identification of saponins, alkaloids, terpenoids, glycosides, phlobatannins, sterols, tannins, flavonoids, anthraquinones and phenols using the method adopted by Meriga et al. (2012).

2.3. Isolation of fatty acid from Galium tricorne seeds

The procedure adopted by Castro and García-Ayuso (1998) was used. In this method about 150–180 g mature seeds of the plant were grinded. Seeds of *G. tricorne* are globose, dark brown and 2.0-3.7 mm in size. Similarly mericarps are dark brown, densely tuberculated and 2–4 mm in size. The grinded sample was placed in a thimble-holder, and the solvent *n*-hexane was added to the distillation flask. The solvent was heated at specific temperature. The heated solvent was reached to the thimble holder containing the sample. Solvent from the thimble holder was unloaded and reached again into the distillation flask. The solvent reached again and again from the thimble holder to the distillation flask. During this process fixed oil or fatty acids were dissolved in the solvent. It was repeated for 12 h or until the extract was cleared. After that the *n*-hexane was evaporated from the dissolved fatty acid.

2.4. Compounds identification by using GC-MS technique

GC-MS analysis of Gt.Crd extract and fatty acids were evaluated for the identification of compound by using an Agilent USB-393752 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) with a HHP-5MS 5% phenylmethylsiloxane capillary column (30 m × 0.25 mm × 0.25 µm film thickness; Restek, Bellefonte, PA). It was equipped with an Agilent HP-5973 mass selective detector in the electron impact mode (Ionization energy: 70 eV). This analysis was checkout by using Elmer Clarus 500 Software Gas Chromatography fitted with capillary column Elite-5MS (5% Phenyl 95% dimethylpolysiloxane). The temperature of oven was set from 200°C to 150°C at the rate of 4°C per minute. The temperature was held for 5 minutes. The inlet and interface temperature were maintained at 250-280°C. The carrier gas helium was used at constant rate of 1.0 mL/minute. A volume of 1.0 µL of sample was injected. Energy of 70 eV was used in electron impact mass spectroscopy. Temperature of ions source and quadruple were kept at 230 to 150°C. The compounds were identified by using NIST library 2005. Various compounds were identified in the plant sample. Furthermore, spectral data was used for the identification of compounds from the Wiley and NIST libraries. For the confirmation, fragmentation pattern of the mass spectra was used with data published in the literature (Stein et al., 2002; Adams, 2007).

2.5. Biological screening

2.5.1. Antibacterial activity

Crude extract and fractions of *G. tricorne* were dissolved in distilled water to make 200 mg/mL stock solutions. The pH of the stock solutions was adjusted between 5 and 7. Sterilization of the extracts was carried out over a membrane filter having diameter of 0.2 mm pore size (Sartorius, Minisart) and kept at 4°C until used. To evaluate the antibacterial activity of the standard drug, Gt.Crd and their fractions, four bacterial strains were used i.e., *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumonia* and *Streptococcus pyogenes*. These bacterial strains were obtained from the Department of Microbiology, Abasyn University Islamabad Campus Islamabad, Pakistan. The bacterial strains were sub cultured on sterile agar media. Inoculum was prepared by using sterile wire loop. After that, cork borer was used and make a diameter of 5 mm wells in the sterilized and solidified agar media. Sterile swab was used for the inoculation of petri plates with bacterial culture. Every petri plate was labelled according to the inoculum. A volume of 100 µL of samples was used and filled every well by using micropipette. Antibiotic (Cefixime) was added into the central well of each petri plate as positive control. After that, all the petri plates were incubated at suitable temperature of 37°C for 24 h. Zone of inhibition (ZOI) of each sample was measured after incubation (Mufti et al., 2012). Triplicate data was obtained of each sample represented as mean ± SEM.

2.6. Cytotoxicity assay

For the cytotoxicity assay, standard procedure of brine shrimp cytotoxicity assay was followed (Meyer et al., 1982).

2.6.1. Hatching techniques

Hatching tray was half filled with already prepared brine solution. Eggs of brine shrimps weighing 50 mg were added into one portion of the hatching tray and kept at temperature of 37°C in the incubator. Samples were kept for 24 h in the incubator. Hatched larvae were reserved under light for maturation for further 24 h. Plant samples were applied on the mature brine shrimps to analyze cytotoxicity.

2.6.2. Procedure

Plant samples weighing 20 mg were dissolved in 2 mL of DMSO to get the concentrations of 10, 100 and 1000 µg/mL. From this solution 5, 50 and 500 µL were transferred into the vials. The vials were kept for some time to evaporate the

solvent. Each concentration was made in triplicate. Similarly, 30 shrimps nauplii were transferred to each vial with the help of pasture pipette. By transferring simulated sea water into each vial to make the volume up to 5 mL. 30 shrimps nauplii were transferred to another vials by adding simulated sea water to make the volume 5 mL. This was taken as negative control. Similarly etoposide (standard drug) was taken as positive control. All the vials were maintained at room temperature for 24 h. Etoposide was used as standard drug. The mortality was calculated by counting the dead brine shrimps in each vial. The following equation (Equation 1) was used for the %age mortality rate of the shrimp.

$$\% \text{ mortality} = \frac{\text{Total number of dead shrimps nauplii}}{\text{Total number of shrimps nauplii}} \times 100 \quad (1)$$

2.7. MTT (Tetrazolium Assay)

Standard MTT procedure was used for the determination of cytotoxicity effect of Gt.Crd extract and its fractions. For this purpose, flat bottomed 96 well micro plates were used (Mosmann, 1983).

2.7.1. Procedure

Minimum essential medium eagle was used and different cell lines were cultured in it. To the media fetal bovine (FBS) 5%, streptomycin 100 µg/mL and penicillin 100 IU/mL were added in 75 cm^2 flasks. These flasks were incubated in 5% incubator at 37°C. Cells were counted by haemocytometer after the trypsinization of the growing cells followed by the dilution with particular medium. The prepared cell culture in the concentration of 6 x 10^4 cells/mL was transferred in 100 µL/well to 96 well plates. Incubated for overnight, medium was discarded and fresh medium in volume of 100 µL was added to it. Similarly, plant samples in concentration of 62.5 125, 250, 500 µg/mL were also added. After 24 h, MTT in volume of 10 µL was added to each well and incubated for further 4 h. Similarly 100 µL of DMSO was also added to every well of the micro plate. By using a micro plate reader at absorbance of 570 nm, the extent of MTT reduction to formazan within cells was figured out. By using the following formula (Equation 2) % inhibition was calculated.

$$\% \text{ Inhibition} = 100 - \frac{\text{Mean OD of test sample} - \text{Mean OD of blank}}{\text{Mean OD of negative control} - \text{Mean OD of blank}} \times 100 \quad (2)$$

2.8. Statistical method used

For the comparison of positive control with test compounds, two-way ANOVA followed by Bonferroni post test were used. Likewise, P value less than or equal to 0.05 was considered significant statistically. Two-way ANOVA followed by Bonferroni post test by using GraphPad were applied for the comparison of positive control with the test groups. SPSS software and Excel sheet were also used for the determination of IC_{50} values. At 95% confidence intervals, the standard error mean (SEM) was calculated.

3. Results

Plant samples were checked out for the presence of secondary metabolites. Secondary metabolites have been found in the Gt.Crd of *G. tricorne* shown in the Table 1.

3.1. Antibacterial activity

The antibacterial effect of *G. tricorne* (Gt.Crd) extract and fractions is shown in Figure 1. Gt.Chf fraction showed better activity against all strains of bacteria used followed by Gt.Crd extract. Gt.Eta fraction exhibited activity

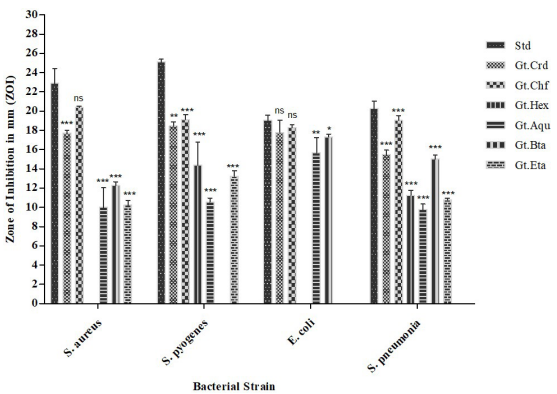

Figure 1. Antibacterial effect of crude methanolic extract of *Galium tricorne* and its fractions. Zone of inhibition = ZOI; Std = standard (Cefixime); Gt = *Galium tricorne*; Crd = Crude; Chf = Chloroform; Hex = Hexane; Aqu = Aqueous; Bta = Butanol; Eta = Ethyl acetate; ns = non-significant; * = p value < 0.05; ** = p value < 0.01; *** = p value < 0.001.

Table 1. Screening of secondary metabolites in *G. tricorne*.

S. No	Secondary Metabolites	Observations	Result
1	Flavonoids	Red or pink red color formation in the solution	**+ve**
2	Saponins	Froth formation	**+ve**
3	Glycosides	Tiny ring brown shaped color formation at the interface	**+ve**
4	Alkaloids	Turbidity, Creamy or Yellow color	**+ve**
5	Phlobatannins	Red colored precipitate formation	**+ve**
6	Tannins	Greenish grey or dark blue color	**+ve**
7	Phenols	Formation of bluish black color	**+ve**
8	Anthraquinones	Pink, red or violet color	**+ve**

against *Staphylococcus aureus*, *Klebsiella pneumonia* and *Streptococcus pyogenes*, but no activity against *E. coli*. Furthermore, aqueous fraction (Gt.Aqu) having activity against all the four strains but less active than Gt.Chf fraction and Gt.Crd extract of *Galium tricorne*. Among all the fractions Gt.Chf fraction and Gt.Crd extract displayed better antibacterial activity. By checking zone of inhibition (ZOI), it was concluded that cefixime presented 25.13 mm inhibition against *Streptococcus pyogenes* followed by the Gt.Chf fraction with 20.37 mm of zone inhibition against *Staphylococcus aureus.* Correspondingly, Gt.Crd extract has 18.47 mm of zone of inhibition against *Streptococcus pyogenes.* The Gt.Aqu fraction reflected moderate activity against all the four bacterial strains. *n*-hexane fraction was inactive against *Escherichia coli* and *Staphylococcus aureus.* It was determined from the obtained results that the plant sample presented antibacterial moiety with greater concentration in chloroform fraction and Gt.Crd extract.

3.2. Brine shrimp cytotoxicity

The brine shrimp lethality effect was measured by using various concentrations of *Galium tricorne* Gt.Crd extract and its fractions. The data was shown in the Table 2. Among all these fractions, Gt.Chf fraction was with outstanding lethality effect in each concentration. The Gt.Chf fraction possessed 88.32, 59.22 and 36.31% cytotoxic effect at concentration of 1000, 100 and 10 µg/mL with LC_{50} of 60 µg/mL. The lethality effect of ethyl acetate fraction (Gt.Eta) was 73.95, 55.57 and 30.60% at concentration of 1000, 100 and 10 µg/mL with LC_{50} value of 80 µg/mL. Similarly, Gt.Crd extract showed the lethality effect of 70.67, 46.67 and 29.00% at concentration of 1000, 100 and 10 µg/mL with 210 µg/mL LC_{50} value. The butanolic fraction of the plant demonstrated lethality effect was 69.67, 45.11 and 22.27% at concentration of 1000, 100 and 10 µg/mL having LC_{50} of 270 µg/mL. The LC_{50} value for *n*-hexane fraction was 330 µg/mL with lethality effect of 58.33, 46.25 and 27.34% at concentration of 1000, 100 and 10 µg/mL. The Gt.Aqu fraction showed low lethality effect 64.44, 39.23 and 19.90% at concentration of 1000, 100 and 10 µg/mL with LC_{50} value of 480 µg/mL.

3.3. MTT assay

From the MTT assay, it has been concluded that Gt.Chf fraction is more active with IC_{50} values of 61 µg/mL and 130 µg/mL against HeLa and NIH/3T3 cell lines respectively followed by Gt.Crd extract with IC_{50} values of 175.46 µg/mL and 167.84 µg/mL against NIH/3T3 and HeLa cell lines. In the same way, Gt.Aqu fraction also carried inhibitory effect with IC_{50} values of 180 and 250 µg/mL against NIH/3T3 and HeLa cell lines. *n*-hexane has been observed with much less cancer cell line inhibitory effect having IC_{50} values of 579.37 and 907.44 µg/mL. The results obtained has been shown in Table 3.

3.4. GC-MS analysis

It is for the first time that GC-MS analysis of various compounds in Gt.Crd extract and fatty acids from the seeds of *G. tricorne* have been detected shown in Tables 4-5 respectively. Spectrum of the compounds is shown in Figure 2. Structures of the bioactive compounds in Gt.Crd extract have been shown in Figure 3. Likewise, spectrum for the identified fatty acids has been displayed in Figure 4 and structural formulae of the bioactive fatty acids identified have been presented in Figure 5.

4. Discussion

In this designed research work, Gt.Crd extract and fractions of *G. tricorne* have been found to possess antimicrobial, cytotoxicity and cancer cell line inhibitory activities, which exhibited hallmark for its medicinal use. Secondary metabolites and detection of compounds in Gt.Crd extract were carried out along with the identification of fatty acids in seeds of the plant. *G. tricorne* is considered novel for the evaluation of such activities. Secondary metabolites identification is also carried for the first time. For this purpose, various chemical tests were employed for the identification of phytochemical in the plant samples. Although, various secondary metabolites have already been isolated from Rubiaceae family which are widely used in folk medicine and showed some analgesic, antibacterial, antiviral, mutagenic, anti-inflammatory, antioxidant and effect on vascular diseases as well as activity on the central

Table 2. Brine shrimp (*Artemia salina*) bioassay of crude extract and fractions of *Galium tricorne*.

Plant sample	No. of Tested Shrimps	Concentrations of the plant sample			LC_{50} µg/mL
		10 µg/mL	100 µg/mL	1000 µg/mL	
		%age death after 24 hours			
Gt.Crd	30	29.00±0.36***	46.67±1.1***	70.67±0.47**	210
Gt.Hex	30	27.34±0.64***	46.25±1.15***	58.33±1.18***	330
Gt.Chf	30	36.31±1.63***	59.22±1.49ns	88.32±0.62ns	60
Gt.BtA	30	22.27±1.30**	45.11±1.35***	69.67±0.94***	270
Gt.Eta	30	30.60±1.35***	55.57±1.27ns	73.95±2.22***	80
Gt.Aqu	30	19.90±1.58***	39.23±2.21***	64.44±1.91***	480

Gt = *Galium* tricorne; Crd = Crude methanolic extract; Hex = Hexane fraction; Chf = Chloroform fraction; Bta = Butanolic fraction; Eta = Ethyl acetate fraction; Aqu = Aqueous fraction. Standard drug - etoposide with LC_{50}=9.8 µg/mL. Data is represented as mean ± SEM, (n=3); ns = non-significant. ** = p value < 0.01. *** = p value < 0.001.

Table 3. MTT assay of crude methanolic extract and fractions of *Galium tricorne* against HeLa and NIH/3T3 cell lines.

Plant sample	Conc. used (µg/mL)	HeLa Cell Line (% Inhibition)	IC_{50} (µg/mL)	NIH/3T3 Cell Line (% Inhibition)	IC_{50} (µg/mL)
Gt.Crd	500	69.49±0.60***	167.84	73.42±0.47***	175.46
	250	56.78±1.36***		65.89±1.97***	
	125	38.92±0.80***		47.29±3.43***	
	62.5	23.44±0.44***		39.44±0.77***	
GT.Bta	500	55.98±1.47***	310.01	41.67±0.93***	745
	250	49.23±1.74***		28.36±0.29***	
	125	35.96±0.73***		11.77±1.58***	
	62.5	24.71±2.33***		7.48±0.35***	
GT.Eta	500	61.69±0.68***	403.02	58.83±0.76***	428.32
	250	42.06±0.97***		45.10±0.51***	
	125	26.61±1.65***		36.23±0.29***	
	62.5	19.16±0.95***		23.47±0.40***	
GT.Hex	500	37.74±1.88***	579.37	24.95±2.20***	907.44
	250	29.67±2.78***		18.44±0.30***	
	125	14.36±0.76***		09.73±1.50***	
	62.5	09.31±0.63***		NA	
Gt.Aqu	500	58.98±0.97***	250	63.04±0.31***	180
	250	49.87±1.42***		57.65±0.45***	
	125	28.74±2.82***		41.13±0.51***	
	62.5	21.15±0.48***		29.57±0.57***	
Gt.Chf	500	79.58±0.65***	61	67.19±0.65***	130
	250	72.78±0.57***		58.68±1.90***	
	125	50.03±1.56***		49.55±0.64***	
	62.5	49.95±0.81***		38.72±0.70***	
Doxorubicin	500	95.51±0.51***	<0.1	97.40±0.80***	<0.1
	250	88.48±1.39***		93.33±0.52***	
	125	84.60±0.40***		87.21±1.85***	
	62.5	81.69±0.36***		85.65±0.73***	

Gt = *Galium tricorne*; Crd = Crude methanolic extract; Bta = Butanolic fraction; Hex = *n*-hexane fraction; Aqu = Aqueous fraction; Chf = Chloroform fraction; NA = non active; Doxorubicin = positive control; IC_{50} has been calculated to be <0.1 µg/ml against both Cell Lines; Data is calculated as mean±SEM; n = 3. *** = p value < 0.001.

nervous system (Heitzman et al., 2005). Antibacterial activity was checked by using well diffusion method for the Gt.Crd extract and fractions of the plant sample. It was concluded that Gt.Chf fraction was more active against all the four bacterial strains followed by Gt.Crd extract as compared to other fractions. Extract and fractions of *Galium tricornutum* subsp. *Longipedunculatum* showed prominent zone of inhibition against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Salmonella typhi* (Jan et al., 2009). *G. tricorne* was also evaluated for brine shrimp cytotoxicity and it was observed that Gt.Chf fraction was more active followed by Gt.Eta and Gt.Crd extract. Through literature, it was revealed that plants related to Rubiaceae family like, *Paederia foetida* L. had significant brine shrimp lethality effect (Morshed et al., 2012). According to a recent report, the positive correlation established between brine shrimp cytotoxicity assay and nasopharyngeal carcinoma of human beings (McLaughlin et al., 1998; Rehman et al., 2009; Fatima et al., 2009). The crude methanolic extract of *Gonzalagunia rosea* Standl belong to Rubiaceae family having strong cytotoxic effect (Niño et al., 2006). HeLa is immortal cell lines derived from cervical cancer cells. In 1951, these cells were taken from late Henrietta Lacks. So HeLa was abbreviated from it (Sharrer, 2006). In 1962 NIH/3T3 cancer cell line was obtained from Swiss mice. These cells are having immortal fibroblast cells and being used widely for experimental purposes (Newbold and Overell, 1983). The crude ethanolic extract of *Rubia cordifolia* roots have significant effect against human cervical cancer cell line (HeLa) and human larynx carcinoma cell line (HEp-2)

Table 4. Identification of compounds in crude methanolic extract of *Galium tricorne*.

S. No	Name of Compound	Retention time	Area	Con. (%)	Molecular formula
1	Ethyl N-hydroxyacetimidate	9.069	62113	0.85	$C_4H_9NO_2$
2	4H-Pyran-4-0ne, 2, 3-dihydro-3,5-dihydroxy-6-methyl-	6.960	133673	1.83	$C_6H_8O_4$
3	2-Methoxy-4-vinylphenol	10.958	32312	0.44	$C_9H_{10}O_2$
4	n-Decanoic acid	12.108	41498	0.57	$C_{10}H_{20}O_2$
5	Dihydroacetinidiolide	16.281	18312	0.25	$C_{11}H_{16}O_2$
6	n-Hexadecanoic acid	16.849	11580	0.16	$C_{16}H_{32}O_2$
7	Heptanoic acid, 2-methyl-2-butyl ester	17.744	203905	2.79	$C_8H_{16}O_2$
8	Tridecanoic acid, methyl ester	20.591	94743	1.30	$C_{14}H_{28}O_2$
9	Tetradecanoic acid	21.331	262776	3.60	$C_{14}H_{28}O_2$
10	Cis-5-Methyl-2-isopropyl-2-hexen-1-al	21.484	67445	0.92	$C_{10}H_{18}O$
11	1-Octadecyne	23.041	50449	0.69	$C_{18}H_{36}$
12	2-Undecanone, 6, 10-dimethyl-	23.144	152927	2.09	$C_{13}H_{26}O$
13	7,10,13-Hexadecatrienoic acid, methyl ester	24.221	30102	0.41	$C_{17}H_{28}O_2$
14	4-Decanoic acid, methyl ester	24.676	22782	0.31	$C_{11}H_{22}O_2$
15	Pentadecanoic acid, 14-methyl-, methyl ester	24.766	740336	10.13	$C_{17}H_{34}O_2$
16	9,12,15-Octadecatrien-1-ol, (Z,Z,Z)-	24.970	107825	1.48	$C_{18}H_{32}O$
17	n-hexadecanoic acid	25.485	2223669	30.43	$C_{16}H_{32}O_2$
18	4-Methyloctanoic acid	26.453	27889	0.38	$C_9H_{18}O_2$
19	Octadecanoic acid	27.329	32556	0.45	$C_{18}H_{36}O_2$
20	9, 12-Octadecadienoic acid, methyl ester, (E,E)-	27.994	348383	4.77	$C_{19}H_{34}O_2$
21	9, 12, 15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	28.114	1032277	14.13	$C_{19}H_{32}O_2$
22	Phytol	28.351	526121	7.20	$C_{20}H_{40}O$
23	8-Dodecen-1-ol, (Z)-	28.748	1083929	14.83	$C_{12}H_{24}O$

Table 5. Identified fatty acids in seeds of *Galium tricorne*.

S. No	Compound labeled	Molecular weight (in gram/mole)	Retention time (in Minutes)	Molecular Formula
1	N-Hexadecanoic acid	256	19.356	$C_{16}H_{32}O_2$
2	Cis-13-Octadecenoic acid	282	21.103	$C_{18}H_{34}O_2$
3	Hexadecanoic acid, ethyl ester	284	19.651	$C_{18}H_{36}O_2$
4	Ethyl Oleate	310	21.303	$C_{20}H_{38}O_2$
5	Butanedioic acid, hydroxyl, diethyl ester	190	3.857	$C_8H_{14}O_5$

(Shyamal et al., 2010). *G. tricorne* was also checked against cancer cell line i.e, HeLa and NIH/3T3 following Tetrazolium assay (MTT). Gt.Chf fraction was with greater inhibitory effect against cancer cell lines followed by Gt.Crd extract and aqueous fraction. Approximately, 3000 different plants possessing anticancer properties were being used as potent anticancer agents. Cytotoxicity effect is the main key for the development of new anticancer drugs and these anticancer compounds have been isolated from the medicinal plants (Kerwin, 2004). Based on the literature, it is realized that the species of the Rubiaceae family like, *Morinda lucida* and *Nauclea latifolia* have potent cytotoxic effects (Karou et al., 2011). Species of the Galium genus like, *Galium verum*, was used in traditional medicine as an anticancer agent. Its effect was checked on head and neck cancer cell lines HLaC78 and proved to be effective in high doses (Schmidt et al., 2014). *Galium aparine* L. also possess anticancer property (Morimoto et al., 2002) and having anti-tumor and immune stimulating activities (Yoon et al., 2008). Various compounds and fatty acids were identified in *G. tricorne* for the first time. Compounds like, diosmetin, isorhamnetin, kaempferol and quercetin

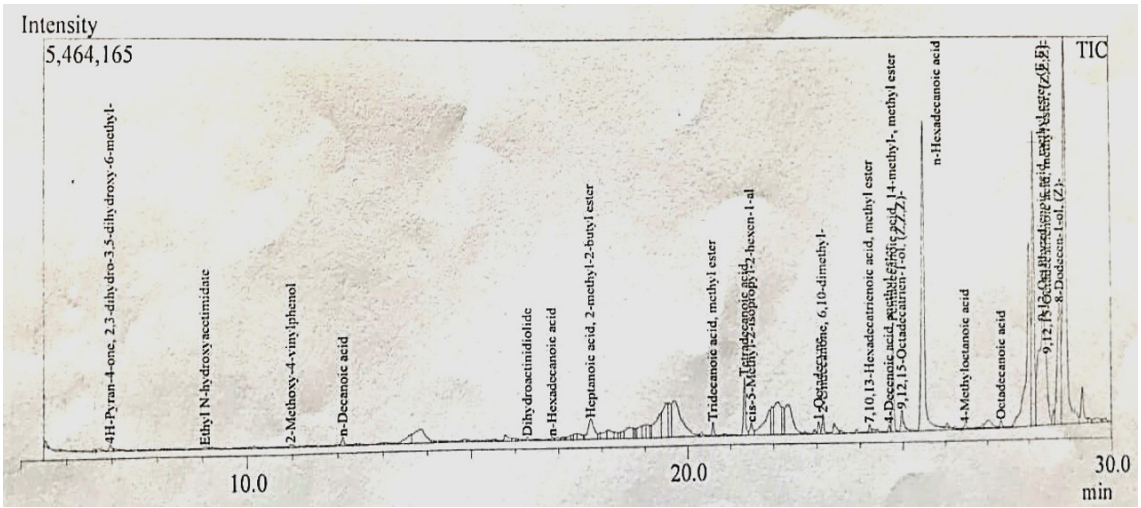

Figure 2. GC-MS analysis of crude methanolic extract of *Galium tricorne*.

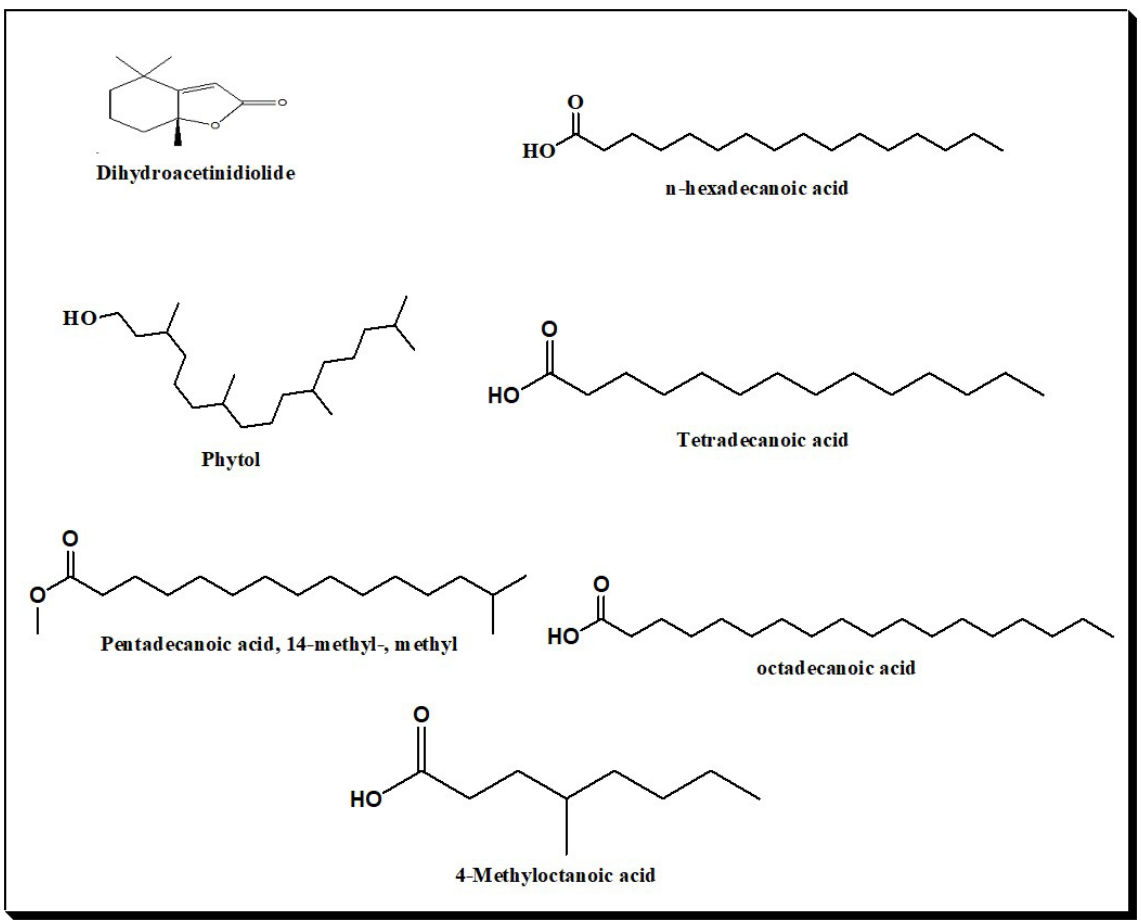

Figure 3. Bioactive compounds isolated from crude methanolic extract of *Galium tricorne* Stokes through GC-MS technique.

were previously reported from *G. verum* (Zhao et al., 2008). Different flavonoids have been reported in *Galium aparine* (Cai et al., 2009).

The compounds identified in Gt.Crd extract and fatty acid identified in *G. tricorne* have been reported for their cytotoxicity effects like, dihydroactinidiolide is a

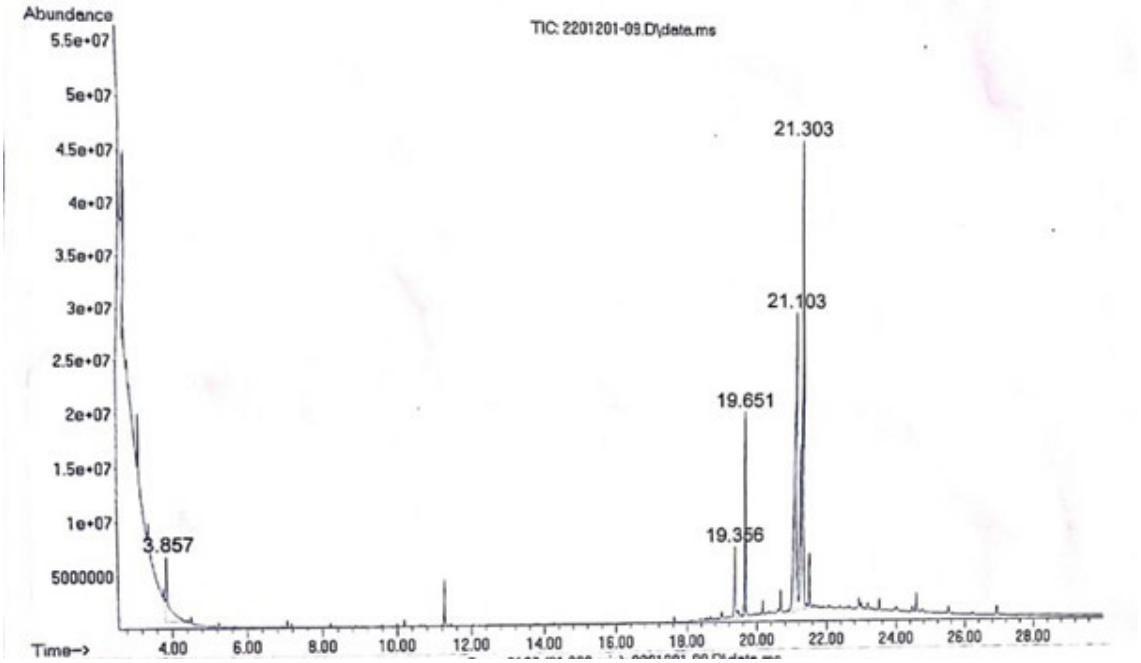

Figure 4. GC analysis of fatty acid from seeds of *Galium tricorne*.

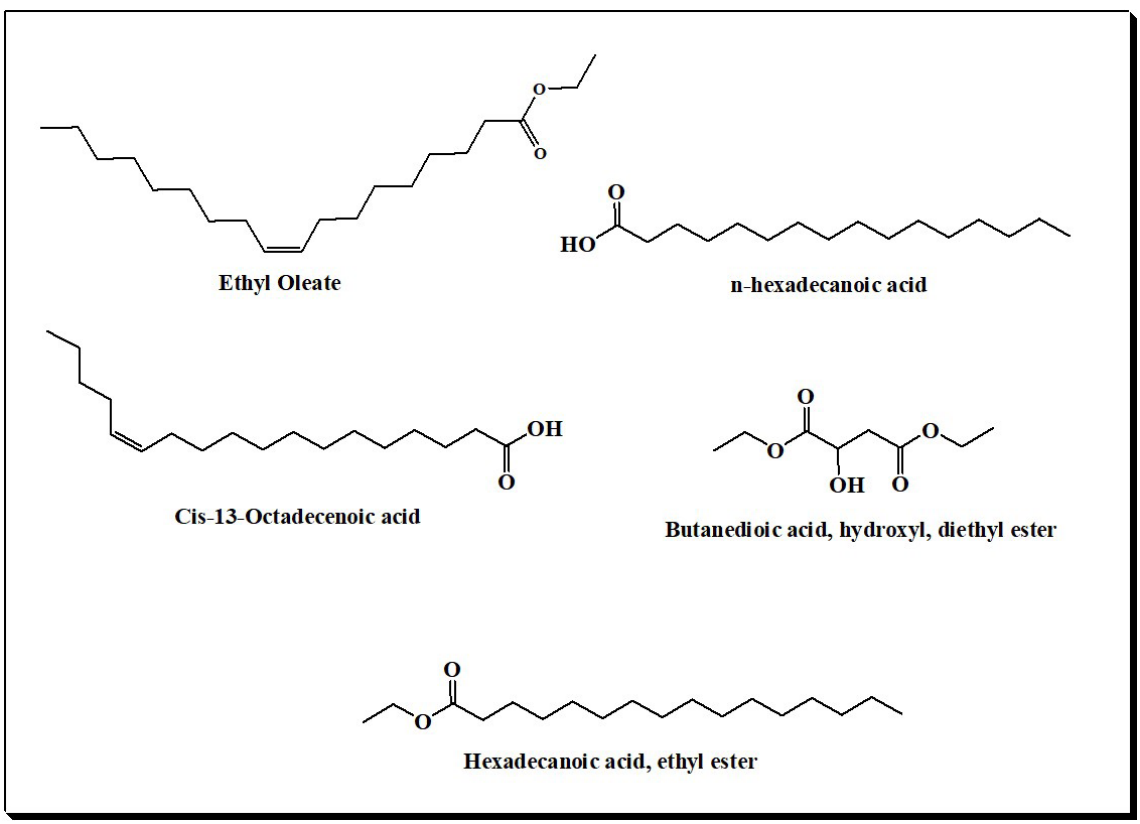

Figure 5. Structures of bioactive fatty acids isolated from the seeds of *Galium tricorne*.

degradation natural product of carotenoids and structure analog of loliolide (Klok et al., 1984; Das et al., 2018). Besides other pharmacological activities, it is well reported with prominent anticancer activity against human tumor cell lines like, human prostate cancer (PC3), hepatocellular carcinoma (HePG-2), epitheliod carcinoma (Hela) and

Mammary gland breast cancer (MCF-7) (Saad et al., 2017). It also induces toxicity against Aβ25-35 in Neuro 2A cells (Das et al., 2018). Phytol has been reported with anticancer potential (Shafie et al., 2015; Zayed et al., 2019). Likewise, Tetradecanoic acid have cytotoxicity against *Culex quinquefasciatus* and *Aedes aegypti* (Sivakumar et al., 2011). The bioactive compound Pentadecanoic acid, 14-methyl-, methyl possess antimicrobial, antioxidant and antifungal potentials (Rangel-Sánchez et al., 2014). It has been proved experimentally that 4-methyloctanoic acid was threefold more potent as compared to Valproic acid for the protection of acute seizure in rates (Chang et al., 2012). N-Hexadecanoic acid has been reported to own antispasmodic, anti-inflammatory antiviral and anticancer activities (Metwally et al., 2020). It also exhibited antimicrobial, ABTS antioxidant and anticancer properties (Madkour et al., 2017). N-Hexadecanoic was also found in *Galium verum* belong to Rubiaceae family (Tava et al., 2020), with antimicrobial and antioxidant effects (Bodoprost and Rosemeyer, 2007). Various compounds like, 1-(ethenyloxyl) octadecane and cis-13-octadecenoic acid has been documented with antioxidant properties (Ajayi et al., 2019). The fatty acid cis-13-octadecenoic acid was served as anti-inflammatory, insectifuge, hypocholesterolemic, anticancer, nematicide, antiarthritic, hepatoprotective, antiandrogenic, antiacne, 5-Alpha reductase inhibitor and anticoronary (Olufunmilayo et al., 2017). It was also reported that cis-13-octadecenoic acid have an important therapeutic role in the treatment of Parkinson disease by preventing dopaminergic cell lose and motor sequelae (Heller et al., 2005). The compound Octadecanoic acid has strong antibacterial activity against *P. aeruginousa* and *B. subitlis* (Sudharsan et al., 2010) and prominent antifungal activity (Shibula and Velavan, 2015). Fruit extract of *Lansium domesticum* having major constituents of ethyl oleate and hexadecanoic acid exhibited cytotoxic effect against KB cancer cells (Manosroi et al., 2012).

From the above discussion, it is clear that the samples of *G. tricorne* were significantly effective and further various bioactive compounds from the Gt.Crd extract of *G. tricorne* have been identified. The antibacterial, brine shrimp cytotoxicity and MTT assay of various plant samples of *G. tricorne* displays the presence of bioactive compounds.

5. Conclusion

Based on the literature review and for the first time biological evaluation and identification of the compounds in *G. tricorne* has been observed. It may be concluded that the aerial part and seeds of *G. tricorne* are the rich sources of bioactive compounds. It may also be inferred that G. tricorne contains a wide variety of anticancer and antibacterial secondary metabolites. Moreover, the results of the current findings also authenticate the scientific background for the ethnomedicinal uses of *G. tricorne*. Furthermore, the toxicity potential of *G. tricorne* might be due to the presence of cytotoxic compounds sorted out in the GCMS analysis.

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