Research Article

Investigation of Phytocompounds and Computational Approach for the Evaluation of Therapeutic Properties of Ethanolic Leaf Extract of *Callistemon citrinus*

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ABSTRACT

Natural products derived from plants, fungi, bacteria and marine organisms are discovered extemporaneously and have a long practice in medicine. Till date, the exploit of natural products, their semi synthetic and synthetic derivatives have been restrained largely to their ethnic use. However, it has been well recognized that each substance has a broad spectrum of biological activities as apparent from some new uses of several old drugs. Prediction of Activity Spectra for Substances (PASS) has been utilized as a potential tool to predict the biological activity spectrum of synthetic substances for the detection of new drugs. The present study was therefore undertaken to examine the biological activity spectrum of the phytoconstituents of *Callistemon citrinus* with their reported biological activities in order to evaluate the applicability of PASS. Subsequently, Molinspiration was utilized to evaluate the oral bioavailability of compounds in order to select the promising compounds that have higher odds of not being discarded in the clinical phase.

Keywords: *C. citrinus*, GC-MS, NIST library, Molinspiration, Osiris, PASS.

INTRODUCTION

Medicinal plants have been providing natural cures for centuries as they synthesize a wide and extensive variety of compounds with therapeutic properties. These compounds have the potential to inhibit the growth of pathogens or to kill them without harming the host cells, therefore they are considered as good candidates for developing new drugs[1]. Medicinal plants are the richest bio-resources of traditional medicines, food supplements, nutraceuticals, pharmaceutical industries and chemical entities for synthetic drugs[2]. The pharmacologically active ingredients obtained from plants have exclusive properties and can be directly used as healing agents[3]. Their mechanism of treating human body is very much similar to the conventional drugs, and they are more preferred as they are safer to use as compared to marketed drugs and more effective in treatment of health problems as they target the biochemical pathways[4]. The study of traditional human uses of plants is an effective way to discover future medicines. On a daily basis extensive studies are being carried out to analyze quality and effectiveness of herbal medicines[5].

Callistemon citrinus (*C. citrinus*) belongs to the family "myrtaceae" and is a momotypic genus. It contains a single species. It is obtained from wet tropics, notably aromatic Australia, South America and tropical Asia, but is now spread all over the world. They are woody aromatic trees and the different parts of this herb have been used in common remedies for treatment of diarrhoea, dysentery and rheumatism.

It is also used as a water accent, anticough, antibronchtits and insecticide in folk medicine. The innumerable medicinal properties and therapeutic uses of *C. citrinus* as well as its phytochemical investigations prove its importance as a valuable plant.

The objective of this study is to list out all the phytocompounds obtained from the different extracts and further assess them with web-enabled bioinformatics softwares such as Molinspiration and Prediction of activity spectra of biologically active substances (PASS) for validation of their therapeutic properties and to justify the use of these leaves for various ailments by traditional practitioners and for future medicines and to validate that the phytocompounds obtained from the extract has better drug efficacy than the commercial drug used for treatment of convulsions.

MATERIALS AND METHODS

Collection of plant materials

The leaves of *C. citrinus* were collected from nursery, VIT University, Tamil Nadu. The leaves were washed with tap water and shade-dried for 3 days.

The leaves were then pulverized to powder in an electric grinder. The resulting 2 kg of sample was stored in an air-tight container for future analysis.

Preparation of plant extract

100 mg of finely grinded powder sample was extracted with 300 ml of ethanol for 3 days. The flasks were kept at room temperature and were stirred at 1 hr interval with the help of a glass rod. After extraction the contents were filtered by Whatmann No.1 filter paper and were concentrated and evaporated to dryness by using a rotary evaporator at 70 °C resulting in a semi-solid viscous mass.

The final residue thus obtained was then subjected to GC-MS analysis[6].

GC-MS analysis

GC–MS (Gas chromatography-Mass spectrometry) is the most preferable tool for the analysis of the volatile chemical compounds in herbal medicines. Both chromatography as a separation method and mass spectrometry provides the benefit as an identification technique[7]. The name, molecular weight and structure of the components of the test materials are ascertained after interpretation on mass spectrum[8].

A Perkin Elmer GC-MS (Model Perkin Elmer Clarus 600, USA) with a VF-5 MS fused silica capillary column (30m x 0.25mm i.d, film thickness 0.25µm) was used to perform the GC-MS analyses of the sample. An electron ionization system with ionization energy of 70 eV was used for GC-MS spectroscopic detection. Pure helium gas was used as a carrier gas at a constant flow rate of 1mL/min[9].

Identification of phytocompounds

Interpretation on mass spectrum was conducted using NIST (National Institute Standard and Technology). It is a database having more than 62,000 patterns. The spectrum of the unidentified component was compared with the spectrum of the known components stored in the NIST library and the retention time (RT), name, molecular weight, concentration (%) and structure of the components were established and taken into account[10]. Each peak obtained from the chromatogram has a repertoire of phytocompounds, which were then analyzed using web-enabled bioinformatics software for their molecular structure, properties and their pharmacological uses.

Prediction of activity spectra for substances (PASS)

With the help of computer program PASS, biological activity prediction of ethanolic extract of *C. citrinus* can be obtained. PASS (Prediction of activity spectra for substances) helps in stating all the pharmacological properties of phytocompounds. PASS works on the basis of structural activity relationship (SAR) analysis[11]. Estimates compounds action in pharmacological effects, interactions with enzymes, toxic effects and effect on gene expression etc[12,13].

Molinspiration

All the compounds were screened, using Molinspiration to test the bioavailability characteristics such as adsorption, distribution, metabolism, elimination (ADME) of the lead compounds. Lipophilicity (miLogP), molecular weight (MW), number of rotatable bonds (NROTB), number of hydrogen bond donors (HBD) and number of hydrogen bond acceptors (HBA) of Lipinski's rule of five were calculated[14,15].

RESULTS AND DISCUSSION

GC-MS analysis

The GC-MS analysis of phytocompounds in the ethanol leaf extract of *C. citrinus* revealed the presence of several phytocomponents. The identification of the phytocomponents was confirmed based on the peak area, retention time and molecular formula (Table 1 and Figures 1-6). The results revealed the presence of following six compounds: (1) eucalyptol, (2) benzamide, 4-fluoro-n,n-dimethyl-, (3) tetracyclo[10.4.0.0(2,11).0(3,8)]hexadeca-3(8),4,6,9-tetraene-2-carbonitrile, (4) 5.beta-pregnane-3.alpha.,20.alpha-diol, bis(trifluoroacetate), (5) 2,4,4-trimethyl-3-hydroxymethyl-5A-(3-methyl-but-2-enyl)-cyclohexe and (6) benz[c]acridine, 5-methyl.

PASS prediction

With the view of finding the specific activity of these compounds, they were exploited for prediction of activity, using PASS. The predicted activity spectrum of a compound is estimated as Pa (probable activity) and Pi (probable inactivity). Prediction of this spectrum is based on the structure activity relationship analysis of the training set, containing more than 205,000 compounds, exhibiting more than 3750 kinds of biological activities. Accordingly, the activities of compounds taken into consideration for possibility of particular pharmacological activity are the ones showing more Pa value than Pi[16]. All the six compounds exhibited a number of biological activities, which included the pharmacological and toxicological activity as in Table 2.

In the present study, PASS predicted that the autoimmune disorders treatment, rheumatoid arthritis treatment, phobic disorders treatment, hepatic disorders treatment, analgesic, antineoplastic (lung, colorectal, colon cancer) activity were expressed by the compound, eucalyptol. Phobic disorders treatment, Taurine dehydrogenase inhibitor, Glutamine-phenylpyruvate transaminase inhibitor, Arylmalonate decarboxylase inhibitor, CYP2C12 substrate activity were expressed by the compound, benzamide,4-fluoro-N-N-dimethyl. Testosterone 17beta-dehydrogenase (NADP+) inhibitor and Antiinflammatory activity were expressed by the compound, tetracyclo[10.4.0.0(2,11).0(3,8)Hexadeca-3(8),4,6,9-tetraene-2-carbonitrile. Membrane integrity antagonist, Dermatologic and Prostate disorders treatment were expressed by the compound, 5.beta-pregnane-3.Alpha.,20,Alpha,-diol,bis(trifluoroacetate). Apoptosis agonist and Antieczematic activity were expressed by the compound, 2,4,4-Trimethyl-3-hydroxymethyl-5A-(3-methyl-but-2-enyl)-cyclohexene.
Thus, the biological activities have been predicted for six phytochemical compounds in the medicinal plant, *C. citrinus*.

Molinspiration

To qualify the compound as a drug candidate there are certain criteria. Lipophilicity (miLogP), molecular weight (MW), number of rotatable bonds (NROTB) was calculated using Molinspiration software. Topological polar surface area (TPSA) is a very valuable constraint for the prediction of drug transport properties[17]. Subsequently, the above mentioned parameters were calculated for all the 6 compounds and the computed molecular properties are depicted in Table 3. All of the phytocompounds were found to violate one of the Lipinski's parameters viz. acceptable. From the data obtained, one can notice that the compounds possess an adequate number of proton acceptor and proton donor groups to ensure efficient interaction with the hydrogen bonding groups of the receptors. The high oral bioavailability is a significant feature for the optimization of bioactive molecules as therapeutic agents. Passive intestinal absorption (associated with MW and logP), low PSA, total hydrogen bond counts (HBA and HBD) or reduced molecular flexibility (measured by NROTB) is essential predictors of good oral bioavailability. Lipinski employed these molecular properties while formulating his Rule of 5 (RO5)[17].

Consequently, the six compounds under study showed moderate to good results when analyzed using Lipinski's RO5. Specifically, the NROTB factor is a measure of molecular flexibility and a very good descriptor of the oral bioavailability of drugs[18]. Rotatable bond is defined as any single non-ring bond, bonded to non-terminal heavy atom (i.e., non-hydrogen). For the compounds in Table 3, 5.beta-pregnane-3.Alpha., 20, Alpha,-diol,bis(trifluoroacetate) had the highest NROTB value (7). 2,4,4-Trimethyl-3-hydroxymethyl-5A-(3-methyl-but-2-enyl)-cyclohexene had three rotatable bonds and Benzamide,4-fluoro-N-N-dimethyl had one rotatable bond. Besides, the calculated PSA values for the compounds ranged from 9.23 Å2 (eucalyptol) to 52.61Å2 (5.beta-pregnane-3.Alpha.,20,Alpha,-diol,bis(trifluoroacetate). According to Kelder, a PSA value less than 60 Å2 tends to identify CNS-active compounds[19]. Thus, the compounds were found to have good penetration into the blood-brain barrier (BBB). However, 5.beta-pregnane-3.Alpha.,20,Alpha,-diol,bis(trifluoroacetate) showed good results in the analysis of Lipinski's RO5, except Nviolations, which was 2, and MW, greater than 500 g/mol (512.53 g/mol), the limits of Lipinski's RO5. These results showed that beta-pregnane-3.Alpha., 20,Alpha,-diol,bis(trifluoroacetate) violates two criteria of RO5, which signified problems with oral bioavailability. Comparatively, 2,4,4-Trimethyl-3-hydroxymethyl-5A-(3-methyl-but-2-enyl)-cyclohexene showed good results in the analysis of Lipinski's RO5 for MW, logP (4.72), TPSA (20.23), natoms (16), Nviolations (0), NROTB (3), volume (248.01), nON(1) and nOHNH(1) counts, thereby indicating its capability to be a potential drug.

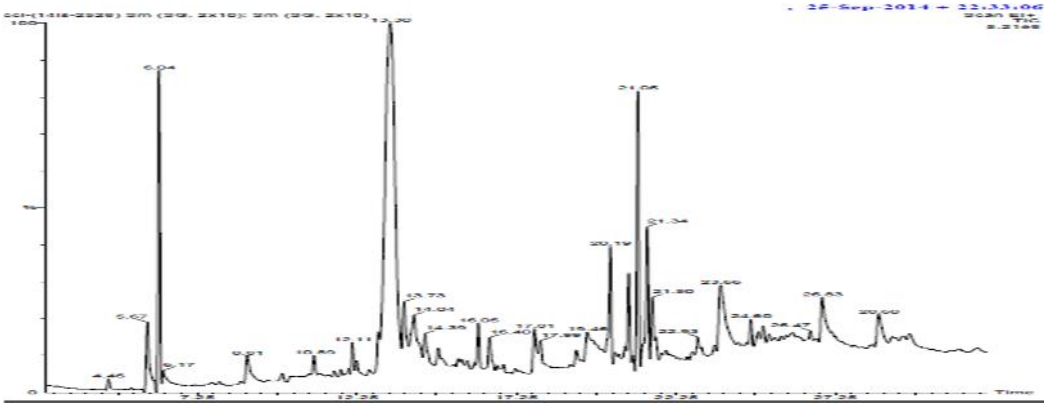

Figure 1: GC-MS chromatogram of ethanol extract of leaves of *C. citrinus*

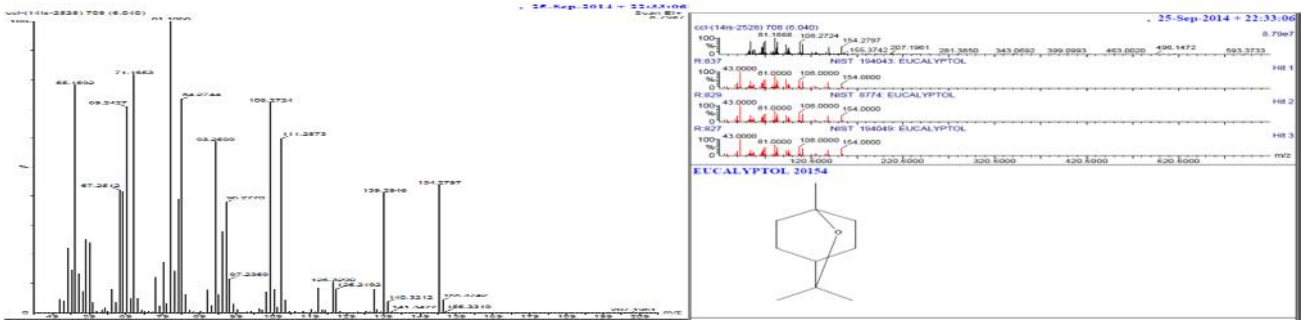

Figure 2: Mass spectrum of Eucalyptol and library search of peak 6.040

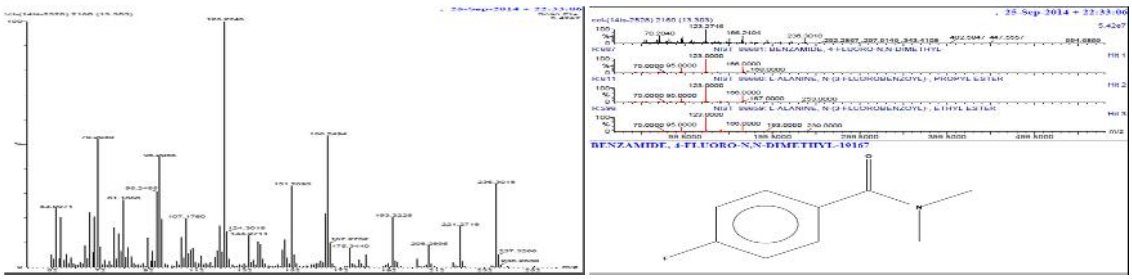

Figure 3: Mass spectrum of Benzamide, 4-fluoro-n,n-dimethyl and library search of peak 13.303

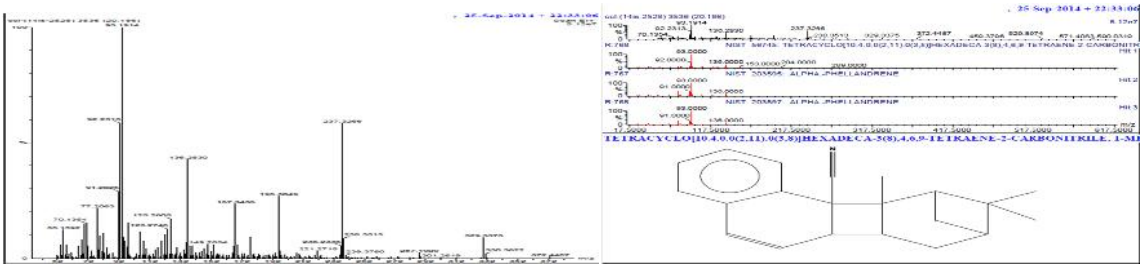

Figure 4: Mass spectrum of Tetracyclo[10.4.0.0(2,11).0(3,8)]hexadeca-3(8),4,6,9-tetraene-2-carbonitrile and library search of peak 20.186

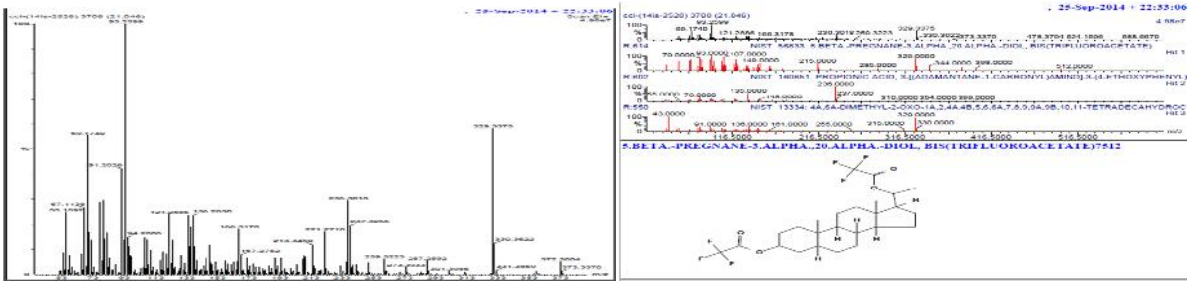

Figure 5: Mass spectrum of 5.Beta-pregnane-3.alpha.,20.alpha-diol, bis(trifluoroacetate) and library search of peak 21.046

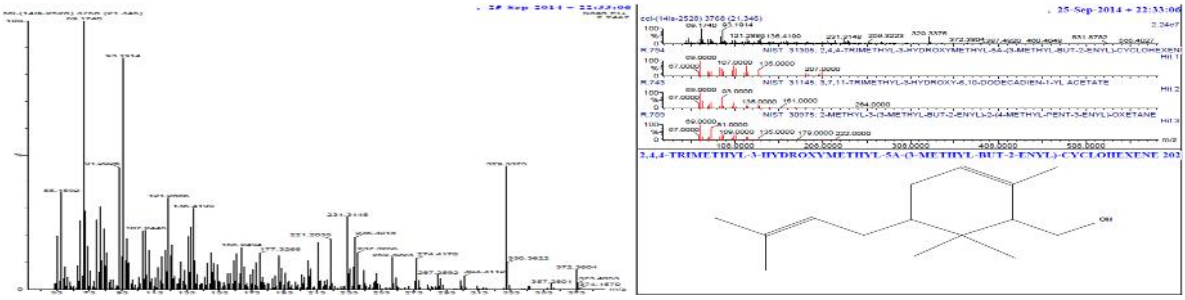

Figure 6: Mass spectrum of 2,4,4-trimethyl-3-hydroxymethyl-5a-(3-methyl-but-2-enyl)-cyclohexene and library search of peak 21.346

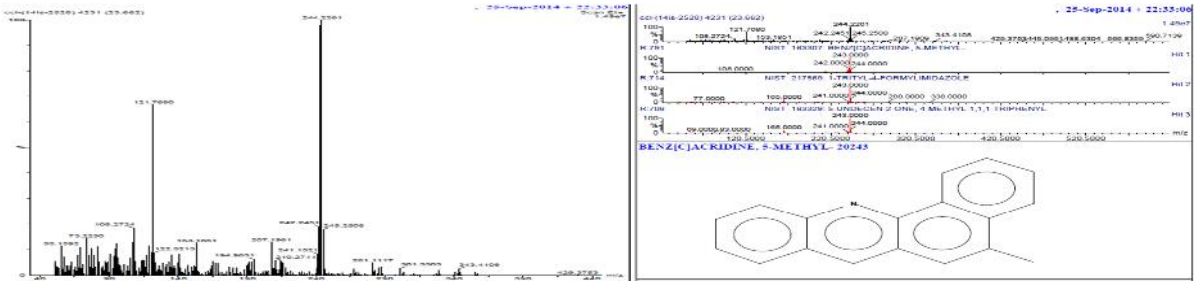

Figure 7: Mass spectrum of Benz[c]acridine, 5-methyl and library search of peak 23.662

Table 1: Phytocomponents in ethanol extract of *C. citrinus* leaves by GC-MS

S. No.	RT	Compound Name	M. Formula	M. wt
1	6.040	Eucalyptol	$C_{10}H_{18}O$	154
2	13.303	Benzamide, 4-fluoro-n,n-dimethyl-	$C_9H_{10}ONF$	167
3	20.186	Tetracyclo[10.4.0.0(2,11).0(3,8)]hexadeca-3(8),4,6,9-tetraene-2-carbonitrile	$C_{21}H_{23}N$	289
4	21.046	5.Beta-pregnane-3.alpha.,20.alpha-diol, bis(trifluoroacetate)	$C_{25}H_{34}O_4F_6$	512
5	21.346	2,4,4-Trimethyl-3-hydroxymethyl-5a-(3-methyl-but-2-enyl)-cyclohexene	$C_{15}H_{26}O$	222
6	23.662	Benz[c]acridine, 5-methyl-	$C_{18}H_{13}N$	243

Table 2: Predicted Pa and Pi values for the GC-MS identified compounds, *C. citrinus* of using PASS

S. No.	Compound Name	Activity	Pa	Pi
1	Eucalyptol	Autoimmune disorders treatment	0,853	0,004
		Rheumatoid arthritis treatment	0,825	0,004
		Phobic disorders treatment	0,833	0,022
		Antiinfective	0,807	0,005
		Acylcarnitine hydrolase inhibitor	0,814	0,012
		Antidyskinetic	0,778	0,007
		5-O-(4-coumaroyl)-D-quinate 3'-monooxygenase inhibitor	0,775	0,011
		Analgesic	0,768	0,005
		Hepatic disorders treatment	0,763	0,004
		Aspulvinonedimethylallyltransferase inhibitor	0,790	0,036
		Antineoplastic (lung cancer)	0,757	0,005
		Antiprotozoal	0,723	0,004
		Nicotinic alpha6beta3beta4alpha5 receptor antagonist	0,740	0,023
		Antineoplastic (colorectal cancer)	0,721	0,005
		Antineoplastic (colon cancer)	0,716	0,005
		Antiseborrheic	0,730	0,032
		Threonine aldolase inhibitor	0,869	0,003
2	Benzamide,4-fluoro-N-N-dimethyl	Phobic disorders treatment	0,806	0,031
		Taurine dehydrogenase inhibitor	0,767	0,018
		Glutamine-phenylpyruvate transaminase inhibitor	0,732	0,008
		Arylmalonate decarboxylase inhibitor	0,701	0,005
		CYP2C12 substrate	0,854	0,023
3	Tetracyclo[10.4.0.0(2,11).0(3,8)Hexadeca-3(8),4,6,9-tetraene-2-carbonitrile	Testosterone 17beta-dehydrogenase (NADP+) inhibitor	0,780	0,031
		CYP2J substrate	0,760	0,031
		Antiinflammatory	0,784	0,008
4	5.Beta-pregnane-3.Alpha.,20,Alpha,-diol,bis(trifluoroacetate)	Membrane integrity antagonist	0,778	0,014
		Dermatologic	0,758	0,005
		Adenomatous polyposis treatment	0,756	0,004
		Prostate disorders treatment	0,752	0,004
		Membrane permeability inhibitor	0,734	0,012
		CDP-glycerol glycerophosphotransferase inhibitor	0,828	0,024
5	2,4,4-Trimethyl-3-hydroxymethyl-5A-(3-methyl-but-2-enyl)-cyclohexene	CYP2J substrate	0,804	0,020
		Apoptosis agonist	0,785	0,009
		Antieczematic	0,777	0,024
		Ubiquinol-cytochrome-c reductase inhibitor	0,764	0,045
6	Benz[c]acridine,5-methyl	Taurine dehydrogenase inhibitor	0,783	0,016

Table 3: Molinspiration table showing values of the parameters involving characteristics such as adsorption, distribution, metabolism, elimination (ADME) of the lead compounds.

S. No.	Compound Name	milogP	TPSA	natoms	MW	nON	nOHNH	Nviolations	NROTB	Volume
Rule	-	<= 5.0	-		<=500	<= 10	<= 5	<= 1		-
1	Eucalyptol	2.72	9.23	11	154.25	1	0	0	0	166.66
2	Benzamide,4-fluoro-N-N-dimethyl	0.92	46.33	12	168.17	3	2	0	1	148.59
3	Tetracyclo[10.4.0.0(2,11).0(3,8)Hexadeca-3(8),4,6,9-tetraene-2-carbonitrile	5.50	23.79	22	289.42	1	0	1	0	285.06
4	5.Beta-pregnane-3.Alpha.,20,Alpha,-diol,bis(trifluoroacetate)	7.20	52.61	35	512.53	4	0	2	7	439.50
5	2,4,4-Trimethyl-3-hydroxymethyl-5A-(3-methyl-but-2-enyl)-cyclohexene	4.72	20.23	16	222.37	1	1	0	3	248.01
6	Benz[c]acridine,5-methyl	4.18	12.89	19	245.32	1	0	0	0	234.61

CONCLUSION

GC-MS analysis of phytoconstituents identified the presence of six different compounds of medicinal importance from the ethanolic leaf extract of *C. citrinus*. Prediction of biological activity of these compounds by using the PASS software exhibited various therapeutic properties. In specific, the Molinspiration calculation suggested that 2,4,4-Trimethyl-3-hydroxymethyl-5A-(3-methyl-but-2-enyl)-cyclohexene satisfies the rule of five, thus indicating favorable bioavailability.

The presence of various bioactive compounds and the confirmation of therapeutic properties rationalize the use of leaf extract for various ailments by traditional practitioners. However, isolation of individual phytochemical constituents and subjecting it to biological activity will certainly offer productive results. Since the leaf extract of *C. citrinus* was found to contain various bioactive compounds, it is suggested as a plant of phytopharmaceutical importance.

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