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Research Article

Therapeutic assessment, chemical characterization and *in-silico* docking of an ethnomedicinal species, *Clerodendrum serratum* (Linn.) Moon.

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Abstract

Clerodendrum serratum (Linn.) Moon (CS) is a traditionally well-accepted plant used vigorously in Indian ayurvedic therapeutic purposes. However, not much work has been carried out to prepare its medicinal profile. Moreover, chemical characterization of this plant is still largely unexplored. In the present study, we explored several *in-vitro* antioxidant methods including hydroxyl radical, nitric oxide, erythrocyte membrane stabilizing activity etc. to evaluate the free radical scavenging potentiality of CS. In addition, GC-MS analysis was performed to identify the active phytocompounds. *In-silico* docking technique was further employed to examine the binding pattern among selected cancerous proteins (breast cancer, ovarian cancer and lung cancer) and identified active compounds. Result exhibited decent antioxidant activity of CS when compared with respective standards. Furthermore, any drastic metabolic changes were not observed through hemolytic assay confirming CS as a non-toxic stuff and safer to consume. A total number of 6 phytocompounds have been identified via GC-MS analysis in which linoleic acid, oleic acid, squalene and stigmasterol are the main bioactive compounds observed. The *in-silico* studies disclosed the probable function of selected phytocompounds in neutralizing various types of cancer leading towards new drug discovery.

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1. Introduction

Oxidative stress induced by reactive oxygen species (ROS) is a major causative agent in the induction of many chronic and degenerative diseases including ageing, diabetes, atherosclerosis, cardiovascular diseases and cancer. Human body is protected from oxidative stress by its own competent defense mechanism; however, the capability of this defensive system is affected by age, diet, food-habit and health status of individual [1]. To maintain a proper equilibrium between ROS and defense system, antioxidants are required as dietary supplement [2,3]. Natural products like, vegetables, fruits and grains, which we consume, have the ability to reduce oxidative damage by acting as antioxidants. Plants have long been utilized as the basis of many traditional medicine exerting protective effects against several diseases due to their antioxidative properties [3]. Antioxidants play a vital role in reducing the progression of cellular damage and cell death in human brain caused by oxidative stress [3]. Besides, overproduction of free radicals in body may also lead to the development of cancer which is

thereby reduced or prevented by the application of antioxidants. In fact, ROS-mediated oxidative stress is the chief causative agent for initiation of several diseases and to counteract the oxidative burst is the main rational pathway for prevention or hindering the pathogenesis. Therefore, there is a growing interest towards use of antioxidants from natural sources.

Clerodendrum serratum (Linn.) Moon, a semi-woody shrub, locally known as '*Bharangi*' belonging to Lamiaceae family, is found mostly in central and South-east Asian Countries as well as the southern part of Africa. Ethnomedicinally, this plant is chiefly used for respiratory complaints viz. colds, bronchitis, bronchial asthma and tuberculosis as it effectively dissolves the mucous [4]. Water decoction of *C. serratum* (CS) has been found to be used to treat high blood pressure in Malayasia [5]. Investigation among Akha people of Thailand and China reflected decent anti-cancerous effect of CS stem [6]. Reviews on CS reported preliminary antioxidant, antibacterial and anti-inflammatory activities [7,8] along with identification of few polyphenolic compound [9]. Furthermore, Chinchali *et al*. [10,11] reported that methanolic extract of CS leaves has considerably reduced tumor development in 7,12-dimethylbenz[α]anthracene (DMBA) induced skin carcinogenicity in testis, liver and kidney of albino mice.

Despite of having decent ethnomedicinal value, no major steps have still been carried out validating the therapeutic relevance of CS. Therefore, an initiative was undertaken to analyze a detail phytochemical profiling as well as therapeutic potentiality of CS. In this regard, free radical scavenging activity of *Clerodendrum serratum* leaf (CSL) was executed in the present study using different *in-vitro* antioxidant methods justifying its beneficial effects over oxidative stress. In addition, Erythrocyte membrane stabilizing activity (EMSA) and haemolytic activity was measured to ensure thesafety and possible cytotoxic mechanism of the CSL, upon consumption. Gas chromatography-Mass spectroscopy (GC-MS) was further employed to identify the bioactive metabolites and their probable functions. Based on the ethnomedicinal importance of CS in cancer treatment [6,10,11], we eventually designed an *in-silico* docking to find out the binding pattern between identified metabolites of CSL and different cancerous proteins including, breast (1n5o), ovarian (2ns2) and lung cancer protein (2j6m) asserting probable anti-cancerous function of CSL.

2. Materials and methods

2.1. Plant material collection and extract preparation

Clerodendrum serratum leaves (CSL) were collected from Guwahati, Assam (26.1445° N, 91.7362° E). The plant material was identified by plant taxonomist and the voucher specimen (Accn. # CS/NBU/ASM/1007) was deposited at the Herbarium of the Botany department. Air-dried (3 weeks) fresh leaves of CSL (13 g) were pulverized into fine powder by using mechanical grinder. The powdered leaves of CLS (10 g) were extracted in a Soxhlet apparatus using absolute methanol (the ratio of plant material to solvent was 1:10 m/v) for 6-7 hours. The extract was then concentrated under reduced pressure and controlled temperature (40-50 ^{0}C) using rotary evaporator (BuchiRotavapor R-3, Switzerland). The extract was further lyophilized using Eyela Freeze Dryer (FDU-506, USA) to obtain dry powder and stored at 4°C until required. The lyophilized CSL extract was dissolved in absolute methanol in desired concentrations each time just prior to use.

2.2. *In-vitro* Antioxidant assays

A total of four *in-vitro* free radical scavenging activity namely DPPH, hydroxyl radical, nitric oxide and hydrogen peroxide as well as phenol and flavonoid content were evaluated to study the efficacy of CSL [12].

2.3. Erythrocyte membrane stabilizing activity (EMSA)

The assay was performed as per the method developed by Concepcion Navarro *et al*. [13] with few changes. Briefly, a RBC suspension was prepared from freshly collected goat blood. Area-ction mixture (1 mL) was prepared containing phosphate buffer (50 mM; 0.2 mL; pH 7.2), distilled water (0.4 mL), RBC suspension (0.1 mL; 10%; diluted in PBS), EDTA (40 µL; 12 mM), 60 µL of nitro blue tetrazolium (NBT; 1%),

riboflavin (40 µL) and varying concentrations of CSL extract (0-200 µg/mL). The reaction mixture was kept under bright light for 30s followed by incubation for 30 min at 50 ^{0}C. Then the mixture was centrifuged for 10 min at 1000 rpm. Finally, the absorbance of the supernatant was measured at 562 nm. Quercetin was used as standard.

2.4. *In-vitro*haemolytic assay

Haemolytic effect of CSL extract was evaluated using freshly collected goat blood according to the standardized method of Malagoli [14] and measured the absorbance of liberated haemoglobin at 540 nm. Triton X-100 was used as positive control.

2.5. GC-MS analysis

GC-MS analysis was conducted as per the standard protocol with slight modifications [15].

2.6. *In-silico* molecular docking

The protein-ligand binding affinity is one of the major criteria to ensure the medicinal property of a particular molecule [16]. Higher binding affinity indicates better effect of ligand on the functionality of protein. Hence, we designed *in-silico* docking to justify the anticancer activity of selected phytocompounds obtained through GC-MS analysis. A few cancerous proteins including breast cancer (1n5o) [17], ovarian cancer (2ns2) [18] and lung cancer protein (2j6m) [19] were chosen for docking analysis. These target proteins were thereby prepared for docking purposes after deletion of water and addition of polar hydrogen. AutodockVina [20] was used for the preparation of both ligand and protein targets. The secondary structures of ligand molecules were downloaded first in .sdf format from NCBI-PUBCHEM (http://www.ncbi.nlm.nih.gov/pccompound). SMI-LES server (https://cactus.nci.nih.gov/translate/) was utilized to convert .sdf format to .pdb format followed by .pdb format to pdbqt and docked with target proteins using AutodockVina software and visualized through PyMol [21].

2.7. Statistical analysis

All the data in the study were prepared as the mean ± SD of six measurements. Statistical analysis was employed by one-way analysis of variance (ANOVA) with Dunnett's test using KyPlot version 5.0 beta 15 (32 bit) for windows, where $p< 0.05$ was considered as significant.

3. Results and discussion

3.1. *In-vitro* antioxidant activities

In the present study, CSL displayed higher free radical scavenging activity (DPPH) of 60.59 ± 0.87% at 200 µg/ml compared to the respective standard ascorbic acid (Fig. 1A). At 200µg/ml of concentration, hydroxyl radical inhibitory activity of CSL and mannitol were found to be 34.39 ± 2.17% and31.31 ± 0.84% respectively (Fig. 1B). Furthermore, Fig. 1C revealed appreciable amount of NO scavenging activity of CSL (58.50 ± 0.02% at 200 µg/ml) in comparison to the standard curcumin. Besides, Fig. 1D revealed considerable potentiality of CSL to quench (43.14 ± 1.37%at 200 µg/ml) H_2O_2 than the powerful standard sodium pyruvate. Immense amount of phenol (67.56 mg gallic acid equivalent per 100 mg of plant extract) and flavonoid content (15.86 mg quercetin equivalent per 100 mg of plant extract) was recorded in CSL.

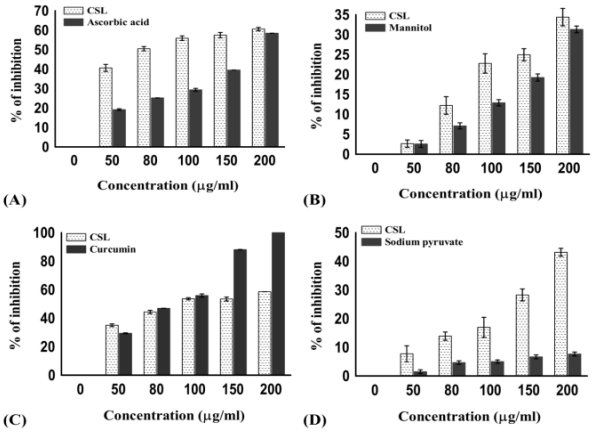

Fig. 1: Free-radical scavenging activities of CSL extract. **(A)** DPPH radical scavenging activities of CSL extract and standard ascorbic acid; **(B)** Hydroxyl radical scavenging capacities of CSL extract and standard mannitol; **(C)** Nitric oxide (NO) scavenging activities of CSL extract and standard Curcumin; **(D)** Hydrogen peroxide (H_2O_2)scavenging activities of CSL extract and standard sodium pyruvate.

3.2. Erythrocyte membrane stabilizing activity (EMSA) and haemolytic activity

We found significant (P <0.001) erythrocyte membrane protective (29.09 ± 0.31% at 200 µg/ml) activity in each dose (Fig. 2A). The CSL extract exhibited significant (P<0.001) lower or negligible

hemolytic activity (Fig. 2B) compared to the positive control Triton X-100 at every dose.

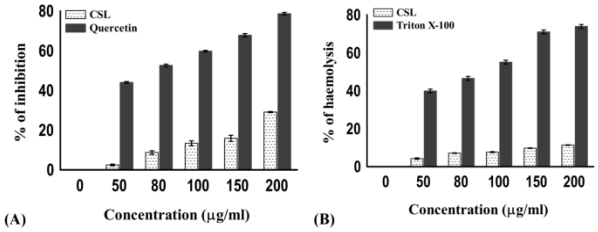

Fig. 2: (A) Erythrocyte membrane stabilizing activity of CSL extract and standard quercetin; **(B)** Haemolytic activity of CSL and standard Triton X-100.

3.3. GC-MS analysis

A total number of 6 phytocompounds have been identified in CSL (Fig. 3) among which linoleic acid (LA), oleic acid (OA), squalene and stigmasterol are the main bioactive compounds observed.

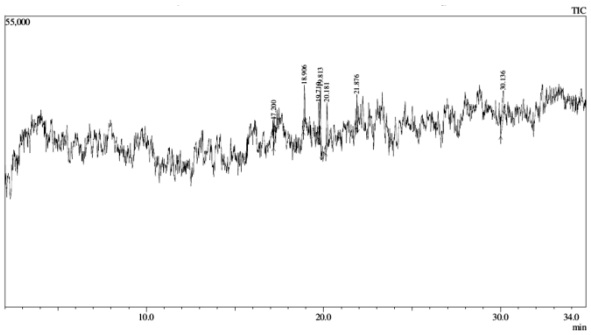

Fig. 3:GC-MS chromatogram ofCSL.

3.4. *In-silico* molecular docking

We observed strong anti-cancerous effect of the identified phytocompounds including stigmasterol and squalene through *in-silico* docking. Result revealed that stigmasterol possesses commendable binding pattern with breast (-6.3 Kcal/mol), lung (-8.7 Kcal/mol) and ovarian cancer proteins (-7.5 Kcal/mol) (Fig. 4A-C) while squalene showed stiff binding affinity (-6.2 Kcal/mol) with the ovarian cancer protein (Fig. 4D).

4. Discussion

Despite of pronounced progress made in themanaging of several diseases using plant-derived phytoconstituents, the quest for plant-based productare still on. Hence, the interest in phenolic and flavonoid substances has gained momentum due to

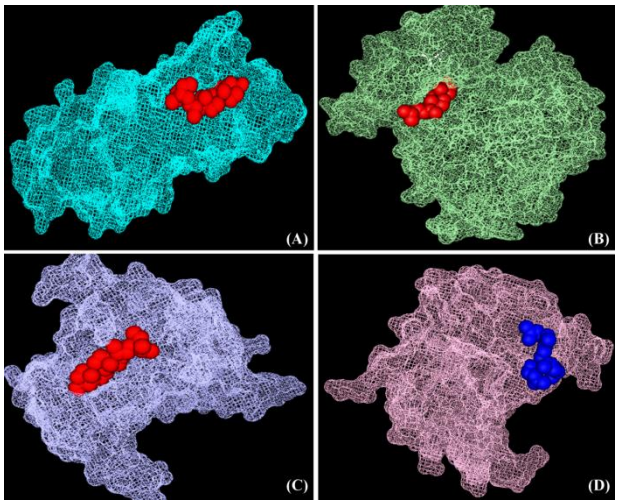

Fig. 4:*In-silico* docking representation of ligands. **(A)** Stigmasterol with breast cancer protein (1n5o); **(B)** Stigmasterol with lung cancer protein (2j6m); **(C)** Stigmasterol with ovarian cancer protein (2ns2); **(D)** Squalene with ovarian cancer protein (2ns2).

their multidisciplinary therapeutic aptitude [22]. In fact, phenolic and flavonoid stuffs are the chief determinants in neutralizing ROS mediated oxidative damage causing most of the life hazardous diseases such as diabetes, cancer, neurological disorders.

Enhanced quantity of phenol and flavonoid content of CSL observed in present study prompted us towards the screening of detail therapeutic appraisal of CSL including free radical scavenging activity, chemical characterization and *in-silico* docking of anti-cancerous activity of identified phytoconstituents.

Free radical scavenging activity through DPPH is a well-established method as this radical is very sensitive to plant active ingredients at low concentration in a very short time. Actually, DPPH radical accepts a proton from any hydrogen donor, mainly from phenolics and become purple to yellow. With the increase of phenolic content in extract, DPPH radical scavenging activity increases and thereby antioxidant activity of respective extract increases [23].

In our experiment, CSL exhibited potent free radical scavenging potentiality than the respective standard (ascorbic acid) attributing its effectual power over oxidative stress. Hydroxyl radical, an extremely

reactive radical, has the capacity to cause damage in almost every molecules found in living cells including DNA, lipid membrane etc. resulting most of the diseases in human. We found that the CSL extract significantly scavenged hydroxyl radicals in a dose dependant manner than the standard mannitol indicating its superior reducing and protective ability. Nitric oxide (NO) radical is another destructive free radical damaging several biological molecules in human body. Upon reaction with superoxide radical, NO gives rise to another detrimental free radical i.e. peroxynitrite ($ONOO^-$). Substantial amount of NO radical scavenging activity of CSL was observed in comparison to the standard curcumin (Fig. 1 C). Concurrently, H_2O_2 accumulating in cells transformed into OH^- in presence of redox active transition metals including Fe^{2+} and Cu^{2+} and produces toxic effect to the cells [24]. Fig. 1D revealed appreciating H_2O_2 inhibitory activity of CSL. However, the inhibition percentage was much lower than the powerful standard sodium pyruvate; it is potent enough to scavenge H_2O_2. Hence, we may propose that the extract has convincing antioxidant activity due to presence of phenolic compounds.

So far the cytotoxicity of CSL extract was concerned upon consumption, erythrocyte membrane stabilizing activity and haemolytic assays were further performed in the present study. Actually, erythrocytes are filled up with hemoglobin consisting of unsaturated fatty acid membrane. Under oxidative stress, superoxide radicals induce the haemolysis of RBC [25]. We found significant (P<0.001) erythrocyte membrane protection activity in every dose (Fig. 2A) asserting CSL as a potent defender of erythrocyte membrane. Meanwhile, hemolysis occurs due to destruction of red blood cells which resulted from lysis of membrane lipid bilayer [25]. Therefore, plant extract are needed to be evaluated for their potential hemolytic activity. Result exhibited negligible hemolytic activity (Fig. 2B) at every dose and thereby the use of CSL is secure to consume. Hence, CSL may be regarded as the new bio-safety candidate to be consumed.

After getting noteworthy results in all aspects of experimentation expressing its potent therapeutic activities, we focused on the identification of active compounds in CSL through GC-MS analysis. A total number of 6 phytocompounds have been identified in CSL (in which linoleic acid (LA), oleic acid (OA), squalene and stigmasterol are the main bioactive compounds having different medicinal properties. LA is one of the essential fatty acids that human need in diet. Deficiency of LA may lead to growth retardation, infertility, skin and kidney degeneration and abrupt changes in fatty acid composition of lipids [26]. Besides, LA has been reported to suppress human tumor [27] and lung tissue cancer [28]. Another metabolite, OA has been reported to have potential protective effect against breast cancer and colon carcinomas in rats [29,30]. In addition, squalene and stigmasterol were reported as potent antioxidants as well as beneficial against several carcinogens [31,32]. Hence, it may be inferred that the CSL extract might be a good source of anti-cancerous stuff.

Since we observed strong anti-cancerous effect of the identified metabolites of CSL, it prompted us to design *in-silico* docking experiments for better understanding the binding pattern of those compounds with some selective cancerous proteins i.e. breast (1n5o), ovarian (2ns2) and lung cancer protein (2j6m) towards new drug discovery. In fact, docking is one of the foremost tools of screening of many drug discovery projects when the structure of the protein is available [33]. Apart from the drug discovery, the utilized biomolecules or metabolites can also be used as ligands to study the biochemical role of a particular target or may beapplied to a range of structural bioinformatics problems including protein function prediction. We found that the identified metabolite, stigmasterol possesses admirable binding pattern with breast, lung and ovarian cancer proteins suggesting its probable role in the management of cancer. Previous reports [34,35] also claimed the anti-cancerous effect of stigmasterol which supports our result for the first time. On the other hand, squalene showed stiff binding affinity with the ovarian cancer protein. This finding is also supported by the previous report of Newmark [36] where squalene was found to be effective to prevent the development of chemically induced cancer. Rao *et al.* [37] reported that squalene can act as anti-carcinogenic agent by inhibiting the aberrant crypt formation and crypt multiplication.

Therefore, the firm binding affinity of stigmasterol and squalene with cancerous proteins symbolizes their effective role as new anti-cancerous drugs.

5. Conclusion

Despite of having massive ethnomedicinal utilization, proper clinical study of CSL was not yet executed. Hence, we intended to explore its different therapeutic applications in the present study. Results reflected that CSL was found to be a potent free radical scavenger and antioxidative agent with non-toxic nature. In addition, a bunch of phytometabolites were characterized having anti-cancerous activity through GC-MS analysis. Furthermore, *in-silico* docking claimed stigmasterol and squalene as convincing ligands that might be imagined as future drug for cancer.

6. Conflict of interest

Authors declare that they have no conflict of interest.

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