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Short Communication Article

Blood coagulation effect of combined extract of *Thymus vulgaris* L. and *Medicago sativa* L.

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ABSTRACT

Thymus vulgaris and *Mediacgo sativa*, are used as a traditional remedy in the treatment of bleeding disorders. Considering their probabilistic coagulation compounds, in an animal study, forty male mice were randomly divided into 5 groups (n = 8) as well as negative and positive control. Coagulation indices include bleeding time (BT), clotting time (CT), and the number of platelets (PLT) were examined on the 13th day of treatment. A significant reduction in the BT and CT tests, as well as a significant increase in PLT in the treated groups was observed. It is concluded that although the *T. vulgaris* and *M. sativa* extracts have a coagulation effect through primary homeostasis and a common pathway of secondary hemostasis, combined extracts are more effective than individual extracts. Moreover, phenolic and flavonoid compounds are the most affecting compounds that affect platelet number and aggregation.

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1. Introduction

Blood coagulation is a process to prevent blood loss during bleeding (Elyasi et al., 2017). In a healthy person, when the blood vessels are damaged, the blood quickly turns into a gel form and a clot forms to prevent bleeding (Norris, 2003). During bleeding, with the activation of factor XII or VII, the blood coagulation system starts, and subsequently thrombin protein is activated and fibrin monomers are formed (Elyasi et al., 2017). So, vascular wall damage and deficiency of coagulation factors and platelets cause various types of bleeding with different severities (Palta et al., 2014). A variety of treatment methods such as gene therapy and replacement with plasma or recombinant factors are used to treat bleeding disorders, which have side effects such as inhibitory antibody production, short half-life, high production cost, and the transmission of viruses such as HIV (Lethagen, 2003; Franchini, 2013; Valentino, 2014; Windyga et al., 2014; Aghili and Zarkesh-Esfahani, 2016; Monroe et al., 2016; Enayati et al., 2017; Akbarzadeh et al., 2019; Fazeli-nasab et al., 2019; Vatandoost et al., 2022b). Herbal medicines are used by different populations (Abdallah et al., 2022) all over the world to treat various disorders. Despite the availability of modern drugs in the treatment of diseases; people's desire for herbal medicines is increasing (Emeka, 2021). These treatment methods are often considered effective and safe medicine due to their natural origin and natural compounds. Natural compounds such as tannins, saponins, glycosides and other phenols are effective in bleeding control (Rehman et al., 2019). *Thyme vulgaris* contains a variety of these secondary metabolites such as polyphenol groups, flavonoids and alkaloids, which affect bleeding in the blood coagulation process (Triratana et al., 1991; Mirza and Baher, 2003; Hosseini et al., 2010; Pawlaczyk et al., 2010; Raaof et al., 2013b; Hosseinzadeh et al., 2015; Klotoe et al., 2017; Rehman

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et al., 2019). It has been demonstrated that tannins and flavonoids have a positive effect on the blood clotting process (Chen et al., 2005; Fauzi et al., 2018). *Medicago sativa* also contains compounds that are effective in blood clotting, including phytoestrogens, Vitamin K, and Vitamin C (Klotoe et al., 2017; Vatandoost et al., 2022a). It was shown that phytoestrogen compounds in *M. sativa* increase plasma estrogen and subsequently increase fibrinogen. Moreover, in the pharmacology studies, *M. sativa* is used to prevent skin hemorrhages and accelerate the blood coagulation process (Karimi et al., 2013; Mancini et al., 2015; Bilen et al., 2016; Zagórska-Dziok et al., 2020). Although the effect of the *M. sativa* and *T. vulgaris* extracts on thrombosis and hemostasis was demonstrated *in vitro* (Klotoe et al., 2017; Vatandoost et al., 2022a), the *in vivo* assay of individual as well as combined extracts will be useful.

2. Experimental

2.1. Sample preparation and extraction

Fresh leaves of *M. sativa* (Fig. 1a) and *T. vulgaris* (Fig 1b) were prepared from farms around Sabzevar, Iran, authenticated by an expert botanist and the voucher specimen was kept in the herbarium of Hakim Sabzevari University (HSUH) with HSUH0209 and HSUH0210 number, respectivly. After identification, the fresh leaves were washed under running water, shade dried, powdered into small pieces, mixed with 70% ethanol (180 g with 470 mL), and placed on a shaker for 48 hours at 1000 rpm. The extracts were filtered and concentrated at 55 °C by rotary evaporation. Extracts were then placed in a drying oven at 40 °C to drive off the ethanol and water excess. The dried extracts were kept at 4 °C and used for further study.

2.2. Experimental design and animal grouping

Forty male NMRI mice (25-30 g, 6-8 weeks old) were purchased from the Animal Center, Royan Karaj, IRAN. The mice were housed under normal laboratory conditions (21 ± 2 °C, 12/12-h light/dark cycle) with free access to standard rodent water and chow. The animals were adapted for 2 weeks before the experiment. Based on statistical analysis with G-POWER software, instruction of Hakim Sabzevari University's animal ethics committee and previously identified effective dose, three groups (n = 8) were designed for dosages of 100, 300, and 100+300 mg/kg/day of *M. sativa*, *T. vulgaris,* and combined extract respectively. Negative and positive control groups (n = 8) were orally administered with 0.3 mL distilled water and 1200 mg/kg/day tranexamic acid, respectively. The present research was performed in accordance with the Guidelines in the Care and Use of Animals and was approved by Hakim Sabzevari University's Animal Ethics Committee (IR. HSU.REC.1399.02).

2.3. Platelet counts test

Platelet count (PC) was performed manually. On the 13th day, each tail tip was punctured, and a drop of blood was collected and smeared on a glass slide. The dried blood smear was incubated with methanol for 3 minutes and stained with Giemsa dye for 15 minutes. After washing and drying at room temperature, platelets were counted from 10 scopes and their mean was recorded as well (Brahimi et al., 2009).

2.4. Bleeding time (BT)

Bleeding time (BT) was measured based on the Dejana method with some modifications (Vatandoost et al., 2022c) on the 13th day. BT was assessed by amputating 2 mm of the tail tip, and the issuing blood was carefully blotted every 15 seconds using the rough side of a filter paper. When no further blood appeared on the filter paper, the number of bloodstains on the filter paper was counted, and BT (seconds) was calculated by multiplying the total number of blood stains by 15.

2.5. Clotting time (CT)

Calculation of the clotting time was carried out based on the method developed by Li and White (Huang et al., 2010; Vatandoost et al., 2022c). On the 13th day, the tail tip was punctured using a scalpel and a drop of blood from the supraorbital vein was collected on a glass slide. The clotting time was recorded between blood collection and fibrin formation.

2.6. Statistical analysis

GraphPad Prism Software (version 9) was used to measure the analysis data. The significance between the two groups was evaluated using an analysis of variance (One-Way ANOVA) followed by Tukey multiple, Games Hawel comparison test, and transformation test. All results are presented as the means ± SEM and are statistically significant at a p value < 0.05.

3. Results and Discussion

3.1. Platelet count test

The results of platelet number (PLT) showed that the mean platelet number in negative and positive control mice are 6.4×10^4 and 35×10^4 cell/µL, while this average in treated mice is 74, 75 and 90 cell/µL for MS300, TV100, and combined extract, respectively. although there isn't any significant change between treated groups, there was a significant increase of 11 and 15 fold for individual and combined groups compared with the control, respectively. Furthermore, up to 2.5 fold incresed PC was observed in treated groups than the positive control (Fig. 2a).

Both individual and combined extracts had a significant increase effect on platelets number. These extracts' flavonoid, phenolic and alkaloid compounds have been the possible cause of increasing platelets. It is supposed that quercetin (El-Newary et al., 2017) is one of the possible reasons for the increase of platelets, through affecting the thrombopoietin (TPO). This hormone, as the main regulator of platelet production, binds to its receptor on the surface of platelets and

Fig. 1. The photograph of **A**) *Thymus vulgaris* and **B**) *Mediacgo sativa*.

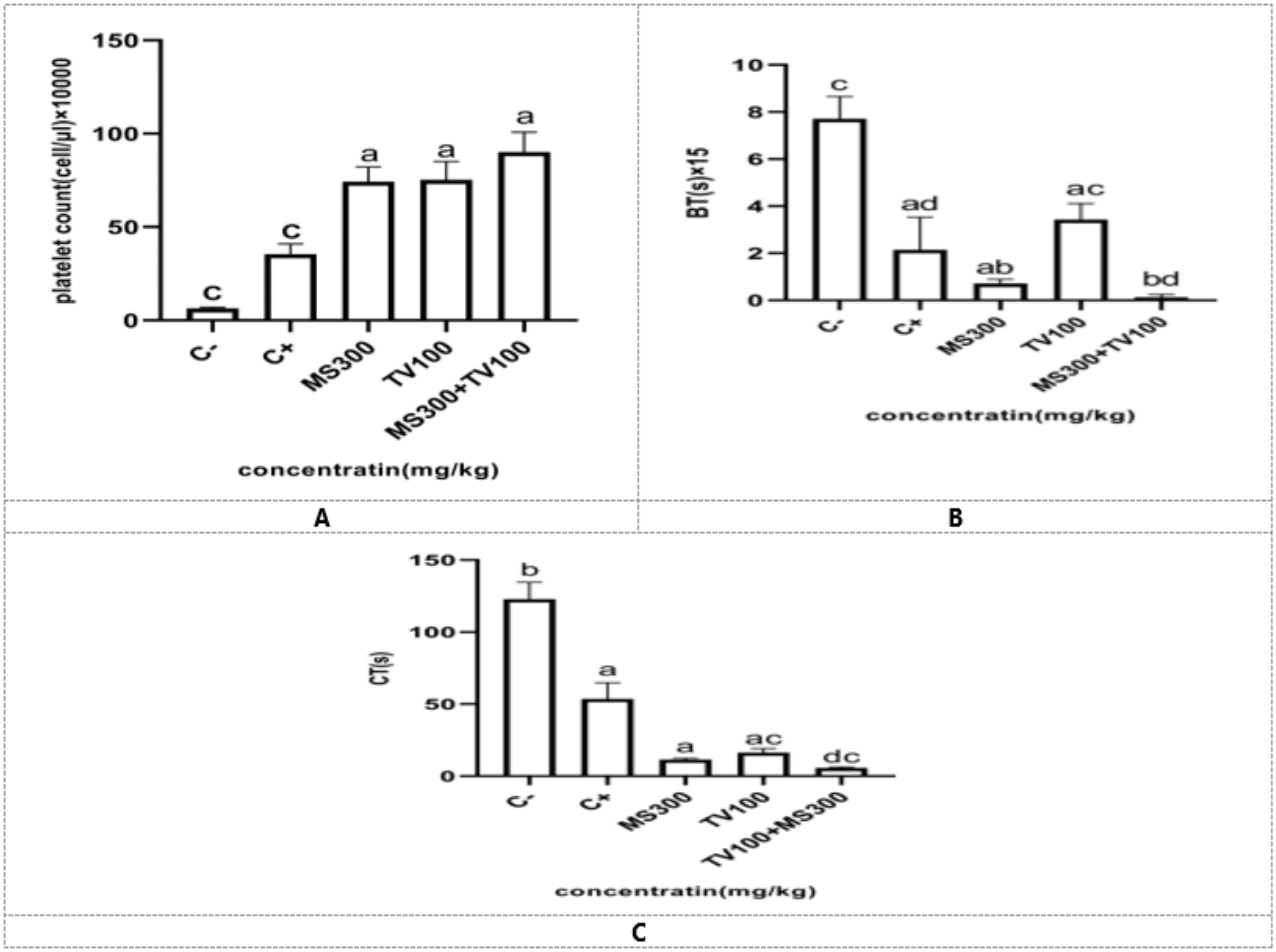

Fig. 2. *In vivo* coagulant effect of the *T. vulgaris* and *M. sativa* extract on blood coagulation parameters. A siginifacant increae in the number of platelets (a) and a significant decreas in bleeding time (b) and clotting time (c) was observed. The data are the means ± SD of three individual experiments, and the significance of the data was shown with different letters.

megakaryocytes, stimulating platelet production. Coumarins can also stimulate the release of interleukin-1-B from human mononuclear cells (Stuhlmeier et al., 1991) which increases the expression of TPO and the production of platelets (Beaulieu et al., 2014). Phenolic acids such as rosmaric acid (Javed et al., 2013) and caffeic acid (Kuete, 2017) can stimulate interleukin-6 secretion from IFN-γ (Radtke et al., 2003), causing an increase in megakaryocyte maturation to platelets. Inhibition of nitric oxide (NO) production in macrophage cells by caffeic acids (Uwai et al., 2008) and coumarin (Peana et al., 2006), suppression of NO production from peritoneal macrophages by Vitamin A and reduction of NO production by retinoic acid in keratinocytes (Bécherel et al., 1996) can lead to an increase in platelets. Polysaccharides can also increase platelets by affecting the runt-related transcription factor 1 (RUNX-1) (Yang et al., 2010) and stem cell factor (SCF) genes (Tajika et al., 1998) that induce megakaryocyte maturation. Since both *T. vulgaris* and *M. sativa* extracts contain compounds such as rosmaric acid, caffeic acid, linalool, coumarin, Vitamin A, tannic acid and quercetin can increase platelet number.

3.2. BT test

The mean value of BT in negative and positive control is 101 and 31 seconds, respectively, while this average time is 11, 51, and 2 seconds in M300, T100, and combined extract, respectively (Fig 2b). There was a significant decrease in BT of the treated groups with M300 and combined extracts compared with the control. In contrast to TV100 which seems not effective, combined extract causes a 50-fold decrease in BT. The BT test, which is related to platelet aggregation and vasoconstriction, is one of the most common tests for the identification of primary homeostasis disorders (Lind, 1991; De Caterina et al., 1994). This test indicates the formation of plaque hemostasis, which depends on sufficient platelet number and adhesion and reduced blood fluidity (Barber et al., 1985). The bleeding time was reduced in the treatment groups compared with the control groups. It is supposed that the inhibition of vasodilators such as nitric oxide can reduce BT. The B vitamins and compounds such as caffeic acid, retinoic acid, vitamin A, linalool, and carvacrol (Asgari, 2013; Kaeidi et al., 2020; Kaidi and Rhahmani, 2020) reduce the production of nitric oxide, result in vasoconstriction, platelet aggregation, and bleeding prevention.

3.3. CT test

The results indicated that the mean value of CT in M300, T100, and the combined extract was 11, 16, and 5 seconds respectively which significantly reduced than control (124 sec). The combined extract caused a 25-fold decrease in CT compared to negative and even 10-fold compared to positive control (Fig. 2c).

The CT test reflects the function of common and intrinsic pathways and platelet aggregation. The coagulation time in the CT test in the treatment groups was significantly reduced compared with the control. It is supposed that beta-carotene increases the number of red blood cells (RBCs), causing high hematocrit and activating platelet aggregation (Pietrzak and Grela, 2015). RBCs are effective in inducing platelet aggregation by releasing a significant portion of their adenosine diphosphate (ADP). Released ADP from RBCs has a 60% and 28% contribution to the reduction of individual platelets and adhesion of platelets, respectively. It has been reported that alkaloids can reduce blood CT by inducing epinephrine (adrenaline) secretion (Singh and Singh, 1975), increasing the FV amount. Both *T. vulgaris* and *M. sativa* extracts contain gallic acid and other flavonoid compounds, which increase the expression of TNF-α (Karimi et al., 2013; Mancini et al., 2015; Bilen et al., 2016; Zagórska-Dziok et al., 2020). TNF-α causes platelet activation by interacting with its receptor on the surface of platelets. Another effective factor may be linoleic acid which causes the accumulation of platelets by producing arachidonic acid (Lee et al., 2003). In fact, arachidonic acid is converted to prostaglandin H2, and this to thromboxane α2 which is the cause of platelet aggregation and vasoconstriction. Tannins are another possible reason for the reduction of clotting time. Tannins can precipitate blood proteins such as albumin, and this process of protein deposition induces the synthesis of thromboxane α2 to increase platelet aggregation and thus accelerate the formation of platelet aggregation at the site of damaged vessels (Fauzi et al., 2018). Vitamin E (alpha tocroferol) is another reason for the coagulant effects found in *T. vulgaris* and *M. sativa* extracts and inhibits urokinase activity in fibrin plates in a concentration-and time-dependent manner (Ogston, 1982). Urokinase binds directly to plasminogen and produces plasmin, which subsequently dissolves the fibrin clot (Bassampoor, 2002; Bahraini, 2020). Increasing the amount of fibrin decreases the clotting time (Hashemi Tayer et al., 2013). In addition to the common factors between the two extracts that cause CT to be reduced, there are exceptions. reducing CT in *M. sativa* may caused by malic acid, malonic acid, oxalic acid and succinic acid compounds (Xuan et al., 2003; Huang et al., 2010; Hashemi Tayer et al., 2013). The reason of decrease in clotting time in the *T. vulgaris* treatment group is the increase in blood LDL caused by the compounds in the extract. LDLs cause the formation of atherosclerotic plaques in the walls of arteries (Abasalizadeh et al., 2017), followed by vascular thrombosis. The deposition of lipids, especially LDL, and their oxidation in the macrophages of the vessel wall cause the greatest change in the inner layer of the arteries (Hamidpour, 2014). On the other hand, the infiltration of LDL into these cells and their oxidation causes more activation of coagulation factors and increase in platelet accumulation. Oxidized LDL (OxLDL) stimulates the production and accumulation of platelet microvesicles and increases platelet aggregation. *T. vulgaris* extract contains polyphenols and polyphenols can stabilize the structure of LDL particles through interaction with apoprotein B (Chen et al., 2005). Stabilization and increase of LDL increases the formation of OxLDL and subsequently OxLDL activates platelets through surface receptors, changes their shape and ultimately increases platelet aggregation (Hamidpour, 2014). Also, phenols can have synergistic effects with other active ingredients

in the extracts. Although vitamin C helps to stabilize LDL by inhibiting the breakdown of lipid peroxides, the synergistic effects of the extract compounds can be another reason for increasing platelet aggregation and reducing CT. It seems that the synergistic effect of vitamin C and flavonoids in *T. vulgaris* extract can increase platelets and subsequently decrease CT. The antioxidant activity of flavonoids through synergism with vitamin E and C and the synergistic effects between genistein (a type of flavonoid) and ascorbic acid confirm these reasons (Chen et al., 2005). During the combined effects of these two plants, the amount of CT decreased significantly compared to the positive and negative control ($p < 0.05$).

4. Concluding remarks

In addition to current therapies for bleeding disorders, herbal medicine can be one of the alternative methods. Although the procoagulant effect of some herbs was investigated, there are various reports of the superior therapeutic effects of combined extracts compared to individual extracts in the treatment of various diseases. Considering probabilistic coagulation compounds in the *T. vulgaris* and *M. sativa*, an animal study was performed to inquire the effect of the combined extract on mice. Considering the combination of *T. vulgaris* and *M. sativa* extract in the combined extract, the amount of effective compounds on clotting time, including tannins (Klotoé et al., 2012; Deng et al., 2019) , alkaloids (Raaof et al., 2013a), vitamin K (Poston, 1964), succinic acid, malic acid, oxalic acid, malonic acid, arachidonic acid (Lynch et al., 1958), linoleic acid and gallic acid increases (Crescente et al., 2009). Each of these compounds reduces clotting time by affecting each blood coagulation pathway.It concludes that each plant extract has unique and different effects in different coagulation pathways. Moreover, the combined extract had superior hemostatic effects compared to the indivitual extract, indicating the unique performance of the combined extract compared to individual treatment methods. On the other hand, it seems aromatic structures, hydroxyl alcoholic and phenolic groups of herbal compounds play a role in interaction with the coagulation factors. Therefore, it can be used as a blood-coagulant herbal medicine with antiseptic properties, although it seems necessary to investigate clinical trials.

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Authors contributions

Conceptualization: Zahra Mashkani and Jafar Vatandoost; Methodology, Data analysis, Investigation, Writing-original draft: Zahra Mashkani and Jafar Vatandoost; Supervision: Jafar Vatandoost; Animal advisor: Toktam Hajjar; Chemical advisor: Behnam Mahdavi. All authors read and approved the final version of the paper.

Conflict of interest

The authors declare that there is no conflict of interest.

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Ethical considerations

The protocols were approved by the Hakim Sabzevari University's Animal Ethics Committee (IR.HSU.REC.1399.002).

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Original Research Article

Anti-inflammatory and analgesic effects of aqueous extracts of *Vitex agnus cactus* L. and *Cymbopogon nardus* L. against carrageenan-induced inflammation in rats

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ABSTRACT

Vitex agnus cactus and *Cymbopogon nardus* are widely used in traditional and conventional medicine as natural anti-inflammatory agents. Within this framework, the current study was undertaken to examine *in vivo* the anti-inflammatory and analgesic effects of aqueous extracts of the leaves and seeds of *V. agnus cactus* and *C. nardus*. In this relation, aqueous extracts were prepared from the leaves and fruits of *V. agnus cactus* and the leaves of *C. nardus*. The inflammatory process was induced using the carrageenan method. The analysis of the obtained results revealed that the aqueous extract of leaves of *V. agnus cactus* exhibited the highest antioxidant content (80.22 ± 11.7 mg GAE/g for TPC, 72.14 ± 9 mg RE/g for TFC, 680 ± 19.6 mg QE/g for flavones and flavonols, 355.33 ± 23.36 mg AAE/g for TAC, 0.33 ± 0.04 mg/mL for IC_{50}-DPPH, and 0.97 ± 0.04 mg/mL for EC50-FRAP) and anti-inflammatory effect with dose-dependent manner. These results suggest that these plants have the potential to alleviate pain and inflammation when used for therapeutic purposes.

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1. Introduction

The use of herbs for medicinal purposes dates back thousands of years, showcasing the enduring importance of herbal remedies in ancient healthcare systems (Crozier et al., 2008; Agrawal and Jain, 2023; Mohammadhosseini and Jeszka-Skowron, 2023). From Traditional Chinese Medicine to Ayurveda in India, and even in ancient Egyptian and Greco-Arabic medicinal practices, herbs played a crucial role in treating various ailments and maintaining health. These herbal traditions were deeply rooted in the knowledge passed down through generations, where women often played a significant role as healers in their communities. In fact, these natural resources have intensively delved into their phytochemical composition for pharmaceutical, cosmetic, nutraceutical, and food purposes (Sayed, 1980; Billowria et al., 2022; Heise et al., 2023). Confirming the potential use of herbal-based products can be challenging, despite their widespread use in treating various human conditions like diabetes, obesity, cancer, microbial infections, and inflammation (Andrade-Cetto, 2009; Singh and Singh, 2009; Saima et al., 2014; Silva et al., 2016; Saleh, 2023).

V. agnus cactus and *C. nardus* are medicinal plants belonging to the Verbenaceae and Poaceae families, respectively (Kaur et al., 2021; Boujbiha et al., 2023). The plants are commonly well-known in Morocco as "Chajarat Mariam'' and "Lwiza Lhamda" for *V. agnus cactus* and *C. nardus*, respectively.

Traditional medicine, such as Ayurveda, Unani, Chinese, Malay, European, Arabic, and ancient Greek medicines, evoked the medicinal utility of medicinal plants such as *Vitex agnus cactus* and *Cymbopogon nardus* (Kamal et al., 2022; Zeqiri et al., 2022). Different ethnopharmacological studies have documented that both plants have been utilized to treat disorders related to the reproductive, digestive, and integumentary systems (Odenthal, 1998;

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Zahid et al., 2016; Niroumand et al., 2018), and also for the treatment of inflammation (Wuttke et al., 2003; Karami et al., 2021). Other pharmacological activities, including analgesic, sedative, and anticonvulsant effects of these plants have been reported in numerous studies (Avoseh et al., 2015; Karami et al., 2021; Tibenda et al., 2022).

The presence of a broad spectrum of biologically active compounds is highly associated with the remarkable therapeutic properties of medicinal plants such as *V. agnus cactus* and *C. nardus* (Adamov et al., 2022; Bayala et al., 2020; Gebashe et al., 2020). New scientific trends are based on the formulation of new drugs using chemically active agents isolated from natural resources (Ousaaid et al., 2022).

Nowadays, phytochemicals present an excellent alternative to chemical agents, which are often associated with moderate to severe side effects (Kamal et al., 2022). Both medicinal plants mentioned above have been identified as natural remedies for various ailments, as documented in different ethnopharmacological studies (Koshta and Sharma, 2023; Lyoussi et al., 2023). The unraveling of the phytochemistry of both plants showed several bioactive compounds with unique beneficial properties against a huge list of chronic diseases (Bayala et al., 2020; Adamov et al., 2022; Gul et al., 2023). In fact, the main phenolic compounds detected in *C. nardus* were *p*-coumaric, ferulic, salicylic, and vanillic acids (Gebashe et al., 2020). In the same context, *V. agnus cactus* contains citronellal, citronellol, and geraniol as the main active components in its essential oil (EO) (Kaur et al., 2021). The EO of *V. agnus cactus* has an excellent antifungal effect against *Candida albicans, C. tropicalis, C. parapsilosis, C. krusei, C. dubliniensis, Aspergillus flavus, A. niger,* and *Penicillium* (Katiraee et al., 2015). Furthermore, it has been found to be effective against numerous cancer cell lines, including breast adenocarcinoma (MCF-7), lung carcinoma (NCI-H292), promyelocytic leukemia (HL-60), and cervical adenocarcinoma (HEP-2) human cell lines (Ricarte et al., 2020). *V. agnus cactus* induces apoptosis by modulating Bcl-2, Bcl-XL, Bax, Bad, FADD, caspase-8, caspase-9, TRAIL R1/DR4, and TRAIL R2/DR5 (Ilhan, 2021). Interestingly, the *V. agnus cactus* extract selectively suppressed cyclooxygenase-2, which is implicated in the inflammatory process (Ibrahim et al., 2021).

The second medicinal plant under study was *C. nardus*, which is extensively utilized in traditional medicine and has been scientifically investigated to validate its biological properties. In fact, from the phytochemical point of view, *C. nardus* has been known to possess various active compounds such as citronellal, geranial, geraniol, citronellol, and neral that were found to be effective against *C. albicans* (CA-ATCC 90028, CA2, CA3, CA4); *C. krusei* (CK-ATCC 6258, CK2, CK3, CK4), *C. glabrata* (CG-ATCC 2001, CG2, CG3, CG4), *C. tropicalis* (CT-ATCC 13803, CT2, CT3, CT4), *parapsilosis complex-C. parapsilosis* (CP-ATCC 22019, CP1), and *C. orthopsilosis* (CO-ATCC 96141, CO1) (De Toledo et al., 2016). *C. nardus* has also excellent antibiofilm and cytotoxic effects against HepG-2 (hepatic) and MRC-5 (fibroblast) (De Toledo et al., 2016). Moreover, the EO of *C. nardus* shows an important anti-inflammatory effect and antiproliferative activity on the prostate cancer cell line LNCaP (Bayala et al., 2020). Each plant possesses a distinctive and complex chemical composition renowned for its pertinent pharmacological activities. The combinations of these components and the development of new formulations could represent a promising approach to create an effective, safe, and stable product with significant effects.

To the best of our knowledge, no comparative study has been conducted to determine the anti-inflammatory and analgesic effects of both studied plants. In this vision, the current work was designed to determine the anti-inflammatory and analgesic capabilities of both plants (*Vitex agnus-castus* and *C. nardus*) using an experimental animal model.

2. Experimental

2.1. Preparation of aqueous extract

The leaves and fruits of *V. agnus cactus* along with the leaves of *C. nardus* were collected from the Rabat region (33°58′09″ N 6°51′26″ W) during 2020. The vegetal matrices were air-dried and powdered using a blender and the obtained powder of each sample was dissolved in water at a 1/10 ratio (10 g/100 mL). The extraction process (maceration) was sustained for 72 h at room temperature. The obtained extracts were then concentrated using a rotary evaporator and subsequently kept at normal conditions until experimentation.

2.2. determination of total phenolic content (TPC)

Quantification was made according to the Folin-Ciocalteu method (Laaroussi et al., 2020), with slight modifications. Briefly, 500 µL of each extract was blended with 500 µL of Folin-Ciocalteu solution (0.2 N) and 400 µL of sodium carbonate (10 *w/w*%) reagent. The mixture was kept for 2 h in the dark. Then, the optical density of the resulting mixture was measured at 760 nm. The obtained results were expressed as milligrams of gallic acid equivalent per gram of extract (mg GAE/g).

2.3. Determination of total flavonoid content (TFC)

Flavonoid content was determined using the aluminum chloride method as previously described (Laaroussi et al., 2020). Briefly, 150 µL of aluminum chloride ($AlCl_3$), 100 µL of sodium nitrite, and 200 µL of NaOH (1.0 %w/w) were added to 100 µL of each extract and then placed in the incubator for one hour. After this period, the optical density was read at 510 nm. The obtained results were expressed as milligrams of rutin equivalent per gram of extract (mg RE/g).

2.4. Determination of flavones and flavonols

Quantification of flavones and flavonols was made using the aluminum trichloride method as previously described by Kosalec et al. (2004). Briefly, 1 mL of

distilled water, 0.1 mL of $AlCl_3$, 1 mL of sodium acetate (50 g/L) were added to 1 mL of each extract. After 90 min of incubation, the optical density was measured at 420 nm. The findings were expressed as milligrams of quercetin equivalent per gram (mg QE/g).

2.5. Antioxidant activity

The antioxidant ability of the different prepared extracts was examined using three complementary assays, namely total antioxidant capacity (TAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and ferric ion reducing antioxidant power (FRAP).

2.5.1. Total antioxidant capacity (TAC)

Total antioxidant capacity was determined by the phosphomolybdenum method (Laaroussi et al. 2020). Briefly, 1 mL of phosphomolybdenum reagent was blended with 100 µL of each extract and then incubated in a bath at 95 °C for 90 min. The optical density was finally read at 695 nm and the obtained results were expressed as milligrams of ascorbic acid equivalent per gram of extract (mg AAE/g).

2.5.2. DPPH assay

The free radical scavenging ability of the extracts under study was determined according to a technique previously described by Laaroussi et al. (2020). Accordingly, the reaction medium consisted of a blend 25 µL of each extract with 875 µL of DPPH solution (63.4 µM). The optical density of the mixture was read at 517 nm after 30 min of incubation in the dark. The percentage of inhibition was determined using the following equation (Eqn. 1):

$$\text{Inhibition}(\%) = \frac{\text{Control absorbance - sample absorbance}}{\text{Control absorbance}} \times 100 \quad \text{(Eqn. 1)}$$

The term IC_{50} for the DPPH assay was deducted from the corresponding percentage inhibition curve.

2.5.3. FRAP assay

Ferric reducing antioxidant power was determined according to the method described previously by Laaroussi et al. (2020). Briefly, the reaction mixture involved combining 1 mL of FRAP solution with 50 µL of each extract, followed by an incubation period of 15 min. After this period, optical density was measured at 593 nm. The obtained results were expressed as the EC_{50} calculated for the expression of findings.

2.6. Design of experiment

2.6.1. Animal handling and housing

Male wistar rats weighing 160 ± 7 g were procured from the animal house breeding center at the Faculty of Sciences Dhar El Mahraz, Fez, and housed there in a typical habitat (25 °C, 55% humidity, and a 12-hour of light and 12-hour of dark). The investigation was planned in compliance with the National Academy of Sciences and the National Institutes of Health's respective "Guide for the Care and Use of Laboratory Animals" documents. The current work has received ethical approval according to the SNAMOPEQ, USMBA, 2017-03.

2.6.2. Ointment preparation

Weighted amounts of the extracts were combined with petrolatum (Vaseline) as an excipient to create an ointment based on the extracts under study. After adding each dose of the excipient, the resulting mixtures, which included two distinct amounts (5% and 10%), were homogenized (Kaur et al., 2021; Boujbiha et al., 2023). Different ointments were prepared by mixing 5 g of each extract with 95 g of vaseline for 5% and 10 g of each extract with 90 g of vaseline for 10%.

2.6.3. Carrageenan-induced rat paw inflammation

The anti-inflammatory effects of *V. agnus cactus* and *C. nardus* extracts were examined using two different routes of administration (Per os and dermal administration) according to the protocol previously described by Winter et al. (1962). The animals adopted for this assay were allocated into 17 groups of 6 rats each. Groups 1, 2, and 3 were treated with *C. nardus* leaf extract at doses of 150, 300, and 500 mg/kg bw. Groups 4, 5, 6 were treated with *V. agnus cactus* seed extract at doses of 150, 300, and 500 mg/kg bw. Groups 7, 8, and 9 received *V. agnus cactus* leaf extract at doses of 150, 300, and 500 mg/kg bw. Group 10, used as a standard, and treated with diclofenac at a dose of 10 mg/kg.
Other groups were treated using ointments containing 5% and 10% of different extracts under study. Group 11 served as negative control receiving Indomethacin 1%. Groups 12 and 13 were treated with ointment based on 5% and 10% of *C. nardus* extract. Groups 14 and 15 were treated with ointment based on 5% and 10% of *V. agnus cactus* seed extract. Group 16 and 17 treated with ointment based on 5% and 10% of *V. agnus cactus* leaf extract.
Before the injection of the freshly prepared carrageenan suspension (1.0%), the right hind leg circumference of all animals under study was measured. The measurement was repeated several times at 3, 4, 5, and 6 h later. We used the following equation (Eqn. 2) to determine the percentage of inflammation inhibition:

$$\text{PI}(\%) = \left((\text{Ct-C0})\text{Control} - \frac{(\text{Ct-C0})\text{Treated}}{(\text{Ct-C0})\text{Control}} \right) \times 100 \quad \text{(Eqn. 2)}$$

Where the terms C0 and Ct respectively account for average circumference of the rat's hind paw before injection and average circumference of the rat's hind paw after carrageenan injection at a specific time.

2.6.4. Analgesic activity

The analgesic activity of the plants under study was examined by adopting the protocol previously described by Hernández-Pérez and Rabanal (2002), using acetic acid (0.7%). Animals were allocated into 5 groups with 6

rats of each. The first one, served as the negative control receiving distilled water. The second group, served as a positive control receiving tramadol. Groups 3, 4, and 5 were received a dose of the following extracts, *V. agnus* leaf extract, *C. nardus* leaf extract, and *V. agnus cactus* seed extract. All animals were administered an intraperitoneal injection of acetic acid (0.7 *v/v*% on saline, 70 mL/kg) 90 min after oral administration. Following a 30 min acetic acid injection that lasted for 5 min, abdominal concentration counts were taken. The formula used to determine the percentage inhibition of abdominal concentrations is as follows:

$$PI(\%) = \left(\frac{Mn - Mt}{Mn}\right) \times 100 \quad \text{(Eqn. 3)}$$

Where Mn and Mt respectively represent average number of abdominal contractions in the negative control group and average number of abdominal contractions in each group treated with extracts or standard.

2.7. Statistical analysis

Two-way ANOVA and the Tukey test were used in the statistical analysis, which was performed using Graph Pad Prism 5 (Microsoft software). Differences were deemed significant at $p < 0.05$.

3. Results and Discussion

3.1. Antioxidants

Table 1 summarizes the obtained results of antioxidant determination of extracts of both medicinal studied plants. The findings indicated that the extract of *V. agnus cactus* was the richest extract in phenolic content with a value of 80.22 ± 11.7 mg GAE/g, followed by the seed extract of the same plant with a concentration of 52.14 ± 6.2 mg GAE/g. Extracts prepared from *C. nardus* registered the lowest phenolic content with a value of 20.11 ± 1.14 mg GAE/g (Table 1). In the same context, the quantification of flavonoids, flavones and flavonols contents of both plants under study revealed that the *V. agnus cactus* extract registered the highest amounts of TFC and flavones and flavonols with values of 72.14 ± 9 mg RE/g and 680 ± 19.6 mg QE/g, respectively. *C. nardus* extract showed the lowest values of TFC and flavones and flavonols with concentrations of 17.02 ± 2.34 mg RE/g and 22.31 ± 3.22 mg QE/g, respectively.
With regard to antioxidant activity, *V. agnus cactus* leaf extract showed excellent antioxidant ability evaluated by three complementary assays (TAC, DPPH, and FRAP) with values of 355.33 ± 23.36 mg AAE/g, 0.33 ± 0.04 µg/g, and 0.97 ± 0.04 mg/g for TAC, IC_{50}-DPPH, and EC_{50}-FRAP, respectively. While, the *C. nardus* leaf extract registered the weakest antioxidant activity examined by TAC with a value of 22 ± 0.18 mg AAE/g, the same extract exhibited an important antioxidant ability examined by DPPH and FRAP assays with values of 0.35 ± 0.10 µg/g and 0.54 ± 0.02 mg/g, respectively. The obtained results from the present study agree with those previously reported in several published reports (Allanto et al., 2022; Hoxha and Pashollari, 2022; Kavaz et al., 2022; Solekha et al., 2022; Tewari et al., 2022). According to a research on the antioxidant capacity of various *Vitex s*pecies, *V. agnus cactus* leaves exhibit a higher antioxidant capacity than other species such as *V. negundo* and *V. trifolia* (Tewari et al., 2022). Boujbiha et al. (2023) reported that the *V. agnus cactus* fruit decoction exhibited considerable antioxidant ability as evaluated by six complementary assays with EC_{50} values of 0.64 mg/mL for DPPH, 0.35 mg/mL for FRAP, 1.03 mg/mL for ABTS, 0.16 mg/mL for β-carotene bleaching, 0.44 mg/mL for metal chelating, and 3.108 mg/mL for TBARS. In the same context, the examination of antioxidant potency of *C. nardus* essential oil showed that the EO at a concentration of 146.66 µg/mL exhibited a percentage of inhibition of DPPH of 62.14% and an IC_{50} DPPH of 102.19±4.2 µg/µg of DPPH, while gallic acid exhibited an IC_{50} DPPH of 0.11 ± 0.04 µg/µg of DPPH (Bayala et al., 2020). Sagala et al. (2023) found that the optimized extract of *C. nardus* registered a higher phenolic content with a value of 78.762 mg GAE/g extract. Within this framework, optimization constitutes an excellent tool to maximize phenolic extraction.

3.2. Carrageenan-induced pad edema test

3.2.1. Per os administration

Fig. 1 displays the obtained results of the percentage inhibition of different doses of the aqueous extract of *C. nardus* on carrageenan-induced edema. It is clearly observed a significant reduction of edema formation by the aqueous extract of *C. nardus* at all doses from the 3rd hour of the assay. The percentage inhibition increases with time, and it appears that the effect of the aqueous extract under study is long-lasting and dose-dependent manner. It can be seen that the dose of 500 mg/kg showed the most prominent activity with an inhibition percentage of 89.17 ± 4.12% compared to the standard drug used in the present study, diclofenac (1.0%) with a percentage of 92.13 ± 4.12% after 6 h. These findings reveal that *C. nradus* has anti-inflammatory effects on the carrageenan-induced inflammatory response.
Fig. 2 displays the obtained results of the anti-inflammatory effect of seeds of *Vitex agnus-castus*. An excellent decrease in pain by *V. agnus cactus* seed extract was observed at all doses under study from the 3rd h of the assay. The percentage inhibition increases with time and is positively correlated with the dose. Doses of 300 mg/kg and 500 mg/kg showed similar percentage inhibition after 5 h. It can be said that the most effective dose was 500 mg/kg with an inhibition percentage near to 80% compared with the standard drug used diclofenac (1%) percentage of 92.95% after 6 h.
Fig. 3 presents the obtained results of the anti-inflammatory effect of the aqueous extract of *V. agnus cactus* leaves on the carrageenan-induced inflammatory response. Treatment of the obtained results showed a significant reduction of pain by aqueous extract of *V. agnus cactus* leaves of all doses under study from the first 3 h of the assay. The anti-inflammatory

Table 1
Bioactive compound quantification and antioxidant ability of different extracts under study.

Extract	TPC (mg GAE/g)	TFC (mg QE/g)	Flavones and flavonols (mg QE/g)	TAC (mg AAE/g)	IC_{50} DPPH µg/g	EC_{50} FRAP mg/g
V. agnus seeds extract	52.14±6.2	19.11±0.17	390±17.3	210.33±4.85	0.52±0.02	1.33±0.07
V. agnus leaves extract	80.22±11.7	72.14±9	680±19.6	355.33±23.36	0.33±0.04	0.97±0.04[a]
C. nardus leaves extract	20.11±1.14	17.02±2.34	22.31±3.22	22±0.18	0.35±0.10	0.54±0.02[ab]

Values in the same column followed by the same letter are not significantly different by Tukey's multiple range test ($p < 0.05$).

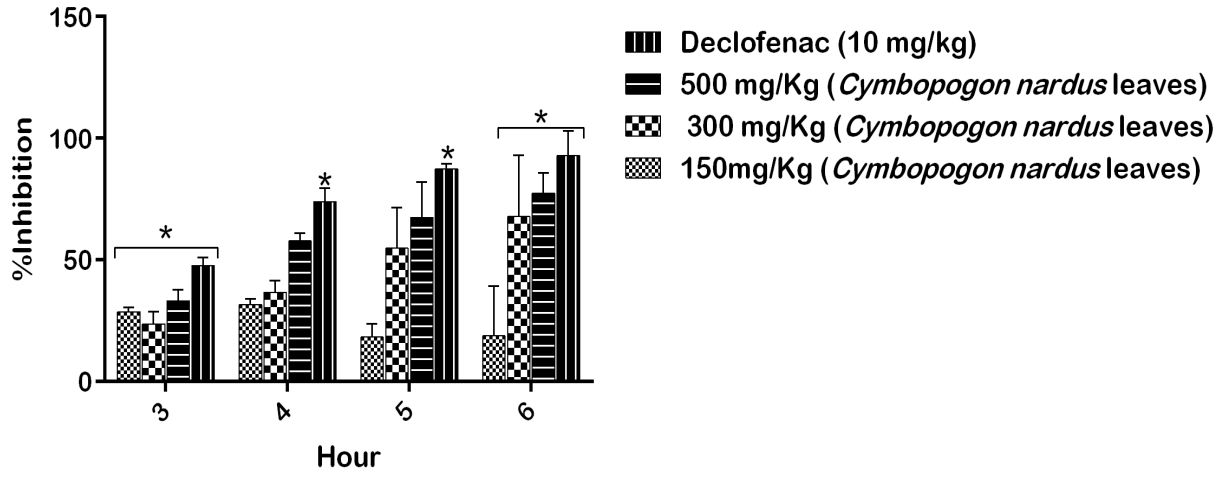

Fig. 1. Inhibition percent of the edema volume after the treatment with the aqueous extract of *Cymbopogon nardus* leaves. The results are expressed as mean±strandard deviation.

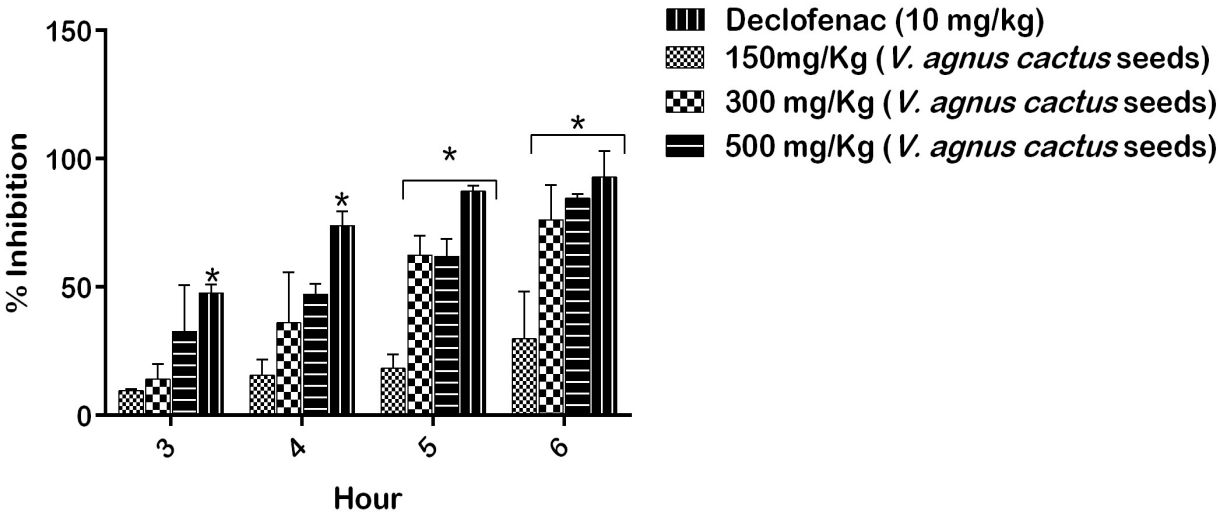

Fig. 2. Inhibition percent of the edema volume after the treatment with the aqueous extract of *Vitex agnus cactus* seeds. The results are expressed as mean±strandard deviation.

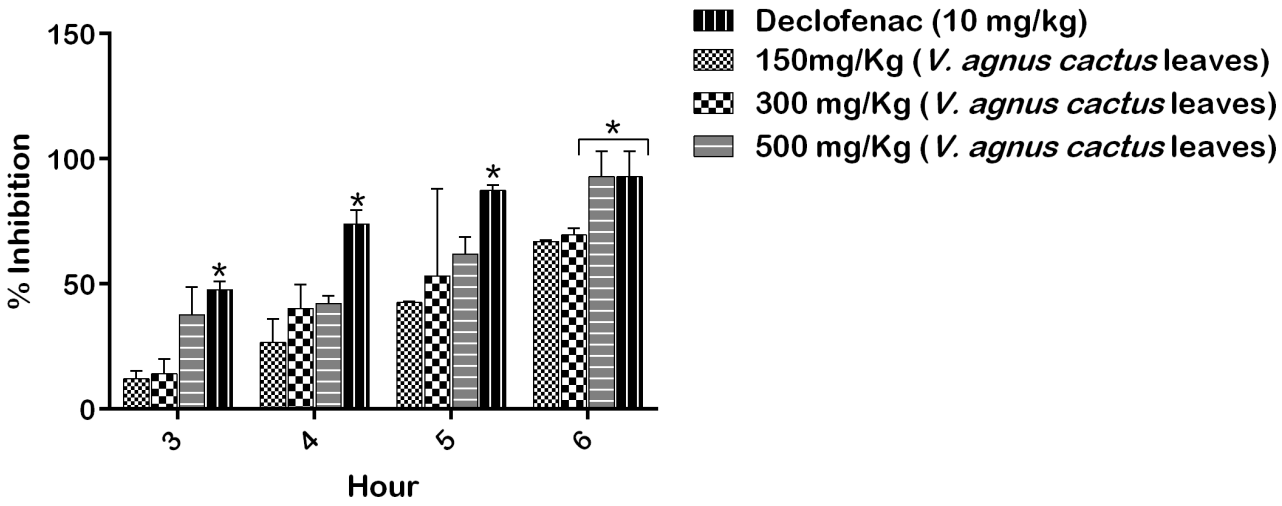

Fig. 3. Inhibition percent of the edema volume after the treatment with the aqueous extract of *Vitex agnus* cactus leaves. The results are expressed as mean±strandard deviation.

effect increases progressively with time, showing a long-lasting. The searching effect is dose-dependent with the dose of 500 mg/kg demonstrating the most potent anti-inflammatory effect and presenting similar inhibition percentage of standard drug used in the present study after 6 h.

3.2.2 Dermal administration

Fig. 4 displays the obtained results of anti-inflammatory effect of the cream prepared using the aqueous extract of *C. nardus*. The analysis of results revealed a significant reduction of edema at both the doses from the 3rd hour of the experiment. It is worth noting that the anti-inflammatory effect of the cream is long-lasting and sustained after 6 h of the assay. When comparing the potency of both doses, it can be shown that the activity is dose-dependent. The most effective effect was shown with a dose of 10% *C. nardus* with an inhibition percentage near 100% compared with the standard drug used (Indomethacin).

The findings shown in Fig. 5 were obtained after using an ointment made from the leaf extract of *V. agnus cactus*. With a percentage time increase to obtain comparable percentage inhibition of the standard drug employed in the current investigation, both dosages produced a notable anti-inflammatory effect.

Application of ointment prepared on the basis of leaf extract showed the results displayed in Fig. 5. As can be seen in this figure, both doses exerted a remarkable anti-inflammatory effect with a percentage time-raising to achieve a comparable percentage inhibition of the standard drug used in this study.

The results shown in Fig. 6 were obtained after applying an ointment produced from seed extract of *V. agnus cactus*. The percentages recorded at various intervals for the two doses used fall short of those recorded for the often prescribed medication.

The analgesic effect of different extracts under study was evaluated using acetic acid at a dose of 50 mg/kg. Fig. 7 displays the obtained results of the Koster assay. Accordingly, it is clearly seen that the administration of different extracts significantly reduced contractions compared with the control. There is no significant difference between all extracts and the standard drug used in this study.

The ability of plants to fight inflammation was closely tied to their antioxidant effect. The highest concentration of antioxidants was found in *V. agnus cactus,* which also had a significant anti-inflammatory effect with a percentage of inhibition comparable to that of the standard drug employed after 6 h of administration (Fig. 3). These findings are in line with those reported by Boujbiha et al. (2023) found that the analgesic and anti-inflammatory effects of *V. agnus cactus* fruit decoction were dose-dependent. *V. agnus cactus* fruit decoction at a dose of 200 mg/kg exhibited a stronger anti-inflammatory effect than the standard drug (lysine acetylsalicylic acid) (Boujbiha et al., 2023). The anti-inflammatory properties of *Haplophyllum tuberculatum* were surpassed by those of *V. agnus cactus* and *C. nardus* (Agour et al., 2022). According to Csikós et al. (2020), using *C. nardus* essential oil reduces inflammatory airway hyperresponsiveness and some cellular inflammatory indicators. Citral, one of the most prevalent bioactive chemicals found in *C. nardus*, has been shown to reduce edema development, histopathological changes, neutrophil activation and adhesion, and the generation of pro-inflammatory indicators (Abe et al., 2003; Shen et al., 2015; Csikós et al., 2020). At a dose of 2.2 mg/mL, *C. nardus* essential oil exhibited a percentage inhibition of lipoxygenase of 25 ± 3% (Bayala et al., 2020).

Carrageenan caused the development of peripheral inflammation in a time-dependent manner, which in turn caused a marked rise in the levels of the inflammatory proteins tumor necrosis factor (TNF), interleukin A (IL-1), nitric oxide (NO), and prostaglandin E2 (PGE2), as well as iNOS and cyclooxygenase-2 protein production in the affected paw (Mansouri et al., 2015). However, administration of the studied plants markedly reduced carrageenan-induced edema. The antioxidants by their

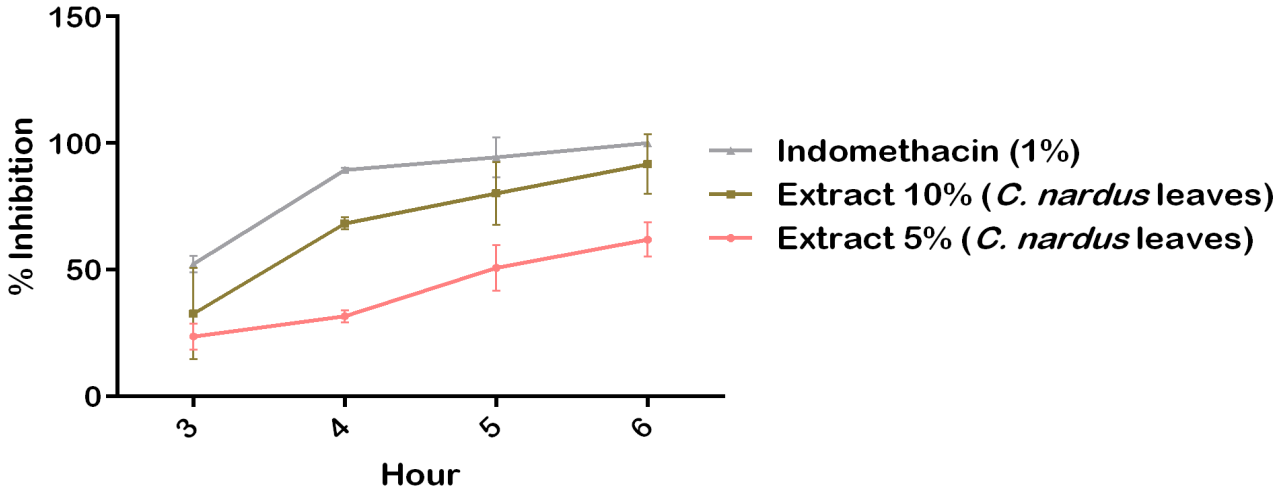

Fig. 4. Inhibition percent of the edema volume after the treatment with the aqueous extract of *Cymbopogon nardus* leaf extract and the standard compound. The results are expressed as mean±strandard deviation.

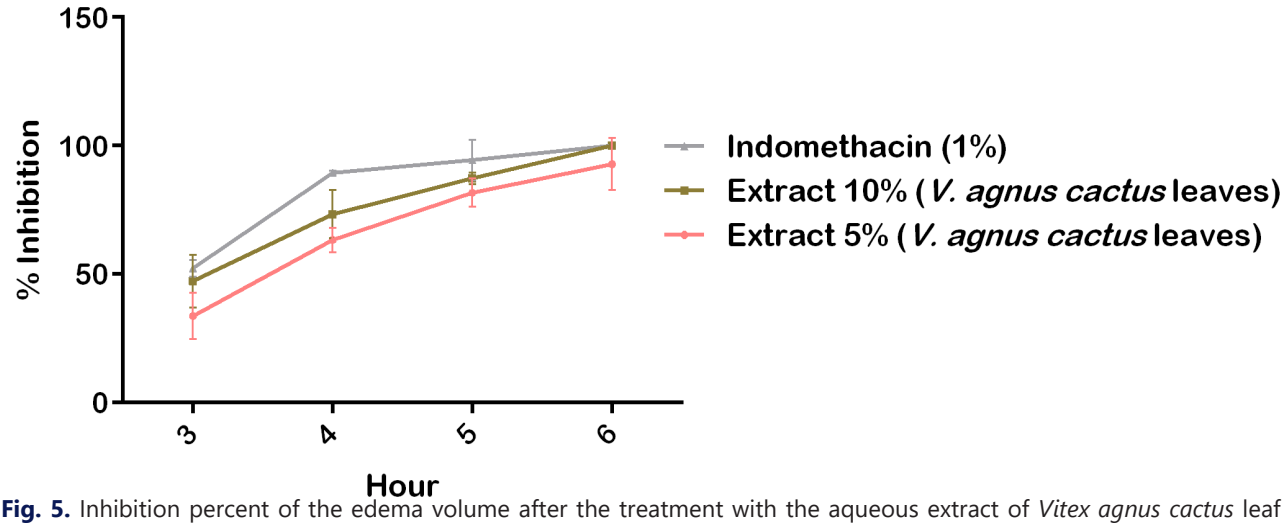

Fig. 5. Inhibition percent of the edema volume after the treatment with the aqueous extract of *Vitex agnus cactus* leaf extract and the standard compound. The results are expressed as mean±strandard deviation.

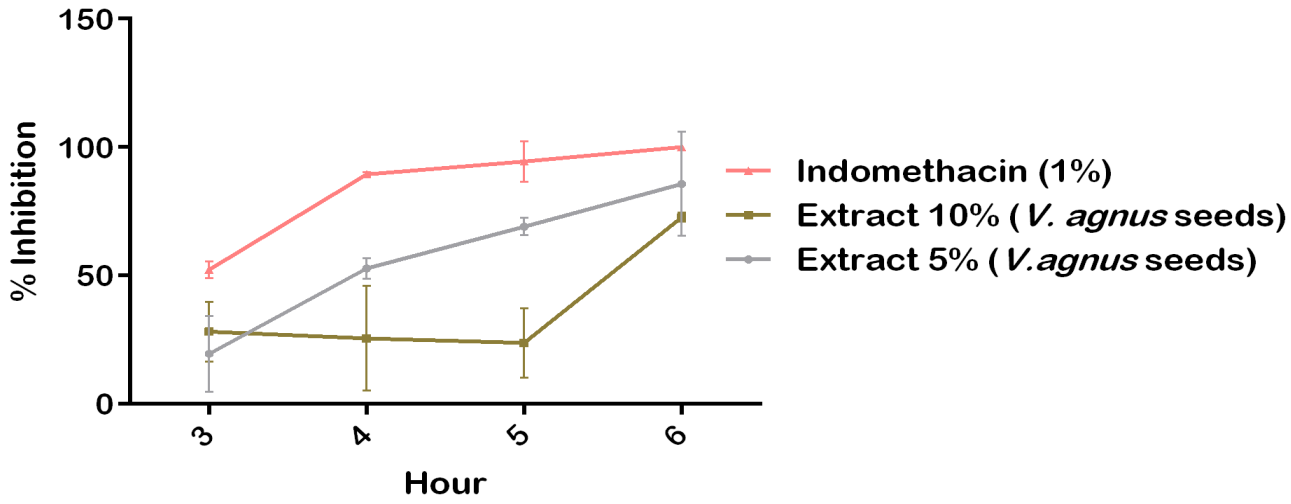

Fig. 6. Inhibition percent of the edema volume after the treatment with the aqueous extract of *Vitex agnus cactus* seed extract and the standard compound. The results are expressed as mean±strandard deviation.

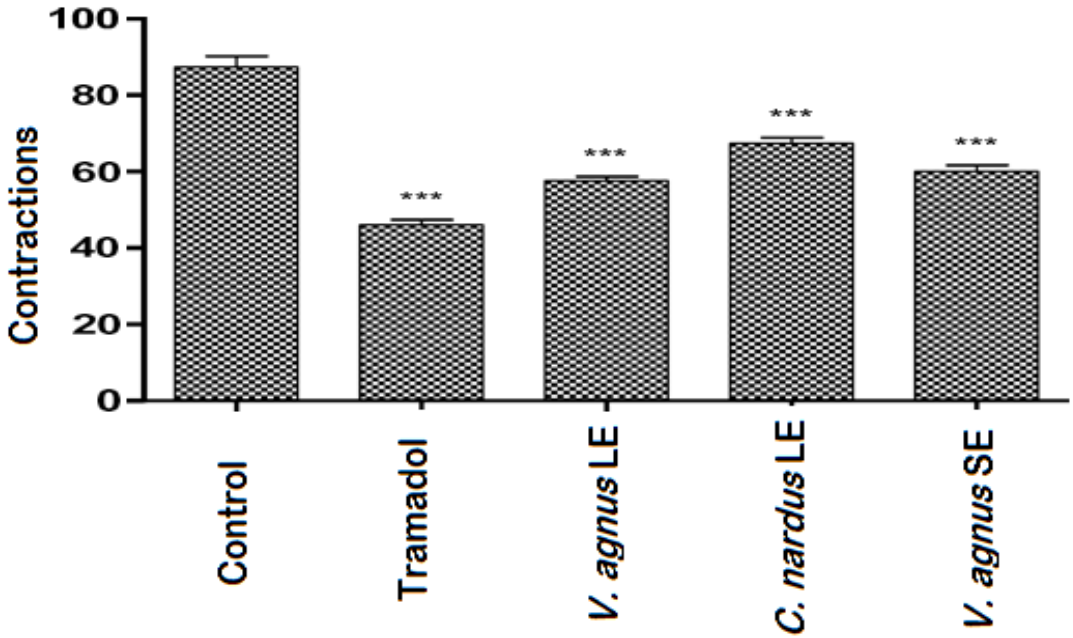

Fig. 7. Contraction inhibition of different extracts under study and the standard compound. The results are expressed as mean±strandard deviation.

pleiotropic effects attenuate the deleterious effects of reactive oxygen species by arresting free radical production and metal chelating, which can achieve anti-inflammation (Chu, 2022). Additionally, they boost the endogenous antioxidant enzymes that are implicated in the ROS elimination process (Chu, 2022). Antioxidants target diverse inflammatory pathways because of their high antioxidant potentials, such as AMPK activation, PI3K/AkT, mTORC1, IKK/JNK, and JAK/STAT inhibition (Yahfoufi et al., 2018). A delve into the phytochemistry of *V. agnus cactus* revealed that 1,8-cineole comprised the highest proportion (30.3%) of the bioactive compounds detected (Boujbiha et al., 2023).

This active component exhibited an interesting anti-inflammatory effect by increasing nuclear factor erythroid 2-related factor 2 (Nrf2) and peroxisome proliferator-activated receptor-γ (PPARγ), whereas, it decreased pro-inflammatory chemokine synthesis (Venkataraman et al., 2023). *In vitro* and *in vivo*, 1,8-cineole inhibits macrophage M1 polarization and prevents HSP90 from suppressing the NLRP3 inflammasome in macrophages (Ma et al., 2023).

Few studies have evoked the anti-inflammatory effect of *C. nardus*. The essential oil of this plant exhibited modest antioxidant and anti-inflammatory activity with a percentage inhibition of lipoxygenase of 0.5 ± 0.9% at a concentration of 0.083 mg/mL compared with gallic acid as the standard active compound used (59.64 ± 2.12%) (Bayala et al., 2020).

The metabolomics profile of *C. nardus* showed the presence of different chemically active compounds like *p*-coumaric, ferulic, salicylic, and vanillic acids, which are well known for their anti-inflammatory effects (Gebashe et al., 2020; Gastelum-Hernández et al., 2023; Song et al., 2023). A plant combination may be a useful product to combat pathogenesis and its complications. To fully exploit the therapeutic potential of medicinal plants and their combinations, additional experimental research is necessary.

4. Concluding remarks

The current comparative study was conducted to investigate the antioxidant, anti-inflammatory, and analgesic effects of two Moroccan medicinal herbs. Our data show that both plant extracts have significant antioxidant, anti-inflammatory, and analgesic efficacy in a dose- and time-dependent manner. *V. Agnus* cactus outperformed *C. Nardus* in terms of antioxidant and anti-inflammatory activity because of its diverse bioactive content. Combining the two plants under consideration can stop or lessen inflammation.

Author contribution statement

Conceptualization and literature search were performed by Fatima El Kamari, Driss Ousaaid, and Badiaa Lyoussi. The first draft of the manuscript was prepared by Fatima El Kamari and Driss Ousaaid. Laila Lahrizi, Abdelfattah El Moussaoui, and Badiaa Lyoussi critically analyzed and gave suggestions to finalize the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that there is no conflict of interest.

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Trends in Phytochemical Research (TPR)

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Original Research Article

Phytochemical analysis of trunk bark with branch bark, wood and stored bark of *Ficus racemosa* and comparison with other *Ficus* barks

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ABSTRACT

The phytochemistry of *F. racemosa* bark was compared with other *Ficus* species available in South India (*F. religiosa, F. benghalensis* and *F. hispida* barks). Further, fresh bark was compared with stored bark of *F. racemosa*; wood with the trunk bark of *F. racemosa*; branch bark with the trunk bark of *F. racemosa*. Also, the protective potential of active fraction was evaluated against oxidative stress in *ex vivo* skin model. Qualitative and quantitative estimation of phytochemicals were done for *Ficus* species bark samples and also subjected to UPLC-QTOF-MS analysis. It was found that methanolic extract of *F. racemosa* has a high number of phytochemicals (Flavonoids: 4.10 mg/mL; phytosterols: 3.28 mg/mL; total phenols: 29.93 mg/mL and tannins: 8.98 mg/mL). Kaempferol-3-*O*-β-D-glucoside was noted as the major compound in *F. racemosa* bark. Trunk bark of *F. racemosa* was quantified to have high amount of phytochemicals than branch bark. Also, bark was found to contain higher concentration of different phytochemicals than *F. racemosa* wood. Stored bark of *F. racemosa* was found to exhibit lesser amount of phytochemicals than fresh bark. *F. racemosa* bark also exhibits moderate superoxide and hydrogen peroxide scavenging activities, high inhibition of lipid peroxidation and moderate inhibition of protein oxidation.

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1. Introduction

The plant *Ficus racemosa*, also known as *Ficus glomerata*, belongs to the family Moraceae. *F. racemosa* commonly called "Cluster fig" in English, "*Aththi*" in Tamil and "*Goolar*" in Hindi, and is distributed in Southeast Asia and Australia (Joy et al., 2001; Ahmed and Urooj, 2010a). It is a lactiferous and evergreen tree that grows to a height of 12 m in damp areas throughout India and is frequently planted for its edible fruit (Kobmoo et al., 2010). The leaves are simple, ovulated; the fruits are grouped together in woody branches; the flowers contain three ovate triangular basal bracts. The bark is reddish-grey in color and appears fractured and thickness of 0.8-1.4 cm (Paarakh, 2009).

In India's traditional medical system, all the parts of *F. racemosa* including the leaves, fruits, bark, latex and sap of the roots are used for various therapeutic purposes. Bark decoction is used to treat ulcers, inflammation, mouth infection, variety of skin conditions, diabetes, piles, dysentery, asthma, gonorrhoea, gleets, leucorrhea, urinary tract disorders and applied as a poultice to swellings and boils (Murti et al., 2010; Ahmed and Urooj, 2011). In Siddha and Ayurvedic systems of medicine, bark of *F. racemosa* is reported as *Attippattai* and *Udumbara*, respectively and is traditionally used for treatment of liver disorders, diarrhoea and respiratory diseases (SPI, 2008). Anti-microbial (Ahmed and Urooj, 2010a), antidiabetic (Keshari et al., 2016), antidiuretic (Ratnasooriya et al., 2003), anti-tussive (Bhaskara Rao et al., 2003), anthelmintic (Chandrashekhar et al., 2008), hepatoprotective activity (Mandal et al., 1999), anti-hyperglycemic (Ahmed and Urooj, 2010b), hypolipidemic (Keshari et al., 2016), antioxidant (Veerapur et al., 2009), anti-inflammatory (Dharmadeva et al., 2018), antipyretic (Bhaskara Rao et al., 2002), anti-diarrhoeal (Mukherjee et al., 1998) and anti-ulcer activities (Rao et al., 2008) of *F. racemosa* bark have also

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been reported. The main flavonoids in the stem bark are bergenin, coumarin, kaempferol, sitosterol, stigmasterol, amyrin acetate, lupeol, lupeol acetate, leucocyanidin-3-*O*-D-glucopyrancoside, leucopelargonidin-3-*O*-D-glucopyranoside and ellagic acid (Ahmed and Urooj, 2010b).

In spite of various medicinal applications, there are some problems associated with *F. racemosa* bark. With respect to distribution and is less frequently available in South India when compared to other *Ficus* species. When market demand for *F. racemosa* bark rises, bark material of other more readily available *Ficus* species such as *F. religiosa* (*Arasamaram* in Tamil), *F. benghalensis* (*Aalamaram* in Tamil) and *F. hispida* (*Peiaththi* in Tamil) could be marketed due to their similar morphology (Bhalerao and Sharma, 2014; Tripathi et al., 2015). As *F. racemosa* bark is cut, immediately dried, made into powder and marketed and if the demand is low, then the bark may be stored, but the data on change in phytochemical profile during storage is not available. Bark is the outer surface layer in the trunk region whereas wood is inner part and wood may also be supplied instead/along with *F. racemosa* bark. Usually the bark from trunk region of *F. racemosa* is being used, but there is a chance to collect the bark from branch also. Due to these alternative materials and variable conditions, the drug marketed in the name of *F. racemosa* bark may cause low efficacy and severe side effects and hence there is a need for comparison of phytochemical profile of related samples with the original drug.

To tackle the problems as stated above, the objectives of the project are devised to analyse and compare the phytochemical constituents of *F. racemosa* bark with other related *Ficus* species (*F. religiosa*, *F. benghalensis* and *F. hispida*); fresh and stored bark of *F. racemosa*; bark and wood of *F. racemosa* & Trunk and branch bark of *F. racemosa*. Further, it is also aimed to isolate and purify the phenolic compounds from *F. racemosa* bark to evaluate its protective potential against oxidative stress in *ex vivo* skin model.

2. Experimental

2.1. Collection and identification of samples

Trunk Bark, branch bark and wood of *F. racemosa* were collected from Medical College campus, Thanjavur, Tamilnadu (10°45′43.0″N & 79°06′19.9″E). Barks of *F. religiosa* and *F. benghalensis* were collected from SASTRA Deemed University campus, Thanjavur (10°43′43.6″N & 79°01′06.2″E). Bark of *F. hispida* was collected from Chettimandapam, Kumbakonam, Tamilnadu (10°58′37.0″N & 79°24′03.6″E) (Supplementary Fig. 1). The identity of the trees was authenticated by Rapinat Herbarium, Trichy and voucher specimen numbers of *F. racemosa* (V.M.L. 001), *F. religiosa* (V.M.L. 002), *F. benghalensis* (V.M.L. 003) and *F. hispida* (V.M.L. 004) were given (Supplementary Fig. 2). The trunk bark materials of all species, branch bark and wood materials of *F. racemosa* (Each sample with ten replicates) were shade dried. Then the materials were chopped and ground with the help of a pulveriser and made into a fine powder using an electric mixer.

2.2. Preparation of extracts

Based on the method of Akshaya et al. (2023), sequential extraction of plant material (Trunk bark, branch bark and wood samples of *F. racemosa* & bark sample of *F. religiosa*, *F. benghalensis* and *F. hispida*) was carried out. A 10 g powdered sample was taken and extracted in 100 mL of solvent with increasing polarity (hexane, chloroform, ethyl acetate, methanol and water). Mixture was shaken for 1 h with continuous stirring using a magnetic stirrer (300 rpm) (Supplementary Fig. 3). The contents were filtered through filter paper and the filtrates were then subjected to further phytochemical analysis.

2.3. Phytochemical screening

All the solvent extracts of barks, branch bark, and wood were tested for the presence of different classes of phytochemicals including alkaloids, flavonoids, terpenoids, phytosterols, saponins, phenols and tannins. To 500 µL of extract, 5 drops of Dragendroff's reagent was added which gives orange-red precipitate to confirm the presence of alkaloids (Sekar et al., 2016). To 500 µL of extract, 500 µL of NaOH added, which turn yellow color indicating the presence of flavonoids (Pant et al., 2017). Formation of red precipitate confirmed the presence of terpenoids when 500 µL of conc. H_2SO_4 was added to 500 µL of extract (Shaikh and Patil, 2020). To 500 µL of extract, addition of 500 µL of CCl_4, 500 µL of CH_3COOH and 500 µL of conc. H_2SO_4 forms reddish brown color in lower layer, which confirms the presence of phytosterols (Shaikh and Patil, 2020). Prolonged foam formation when 500 µL of distilled H_2O was mixed with 500 µL of extract, which indicated the presence of saponins (Santhi and Sengottuvel, 2016). To 500 µL extract, 500 µL of $FeCl_3$ (5%) was added to form dark blue color solution, which confirms the presence of phenols (Deyab et al., 2016). To 500 µL of extract, addition of 500 µL of $Pb(C_2H_3O_2)_2$ (1.0%) formed creamy precipitate, which confirms the presence of tannins (Ukoha et al., 2011). The qualitative results are expressed as + for the presence in low amounts, ++ for the presence in medium amounts, +++ for the presence of high amounts and − for the absence of phytochemicals.

2.4. Quantitative analysis

Total alkaloid content was estimated using the method of Sreevidya and Mehrotra (2003). To 500 µL of different extracts, 500 µL of Dragendroff's reagent was added. The precipitate formed was centrifuged for 5 minutes. Then, the supernatant was decanted completely and the precipitate was washed with 1000 µL of methanol and centrifuged for 5 minutes. The supernatant was discarded and the residue was then treated with 1000 µL of disodium sulphide solution (1.0%). The brownish-black precipitate formed was then centrifuged for 5 minutes and the precipitate was dissolved in 1000 µL of concentrated nitric acid. This solution was diluted with 1000 µL of distilled water, from which 500 µL

was then pipetted out and mixed with 1000 µL of thiourea solution (4.0%). The absorbance was measured using spectrophotometer at 600 nm against the blank containing nitric acid and thiourea. A standard calibration curve for berberine in the range of 100-1000 µg/mL was prepared in the same manner and results were expressed as mg/mL of extract.

Total flavonoid content was determined by following the method of Ahmed and Iqbal (2018). To 100 µL of each extract, 100 µL of sodium nitrate solution (5.0%) was added and incubated at room temperature for 5 minutes. Then, 100 µL of aluminium chloride (1.0%) was added, incubated for 5 minutes and further 700 µL of NaOH was added and incubated at room temperature for 5 minutes. Finally, 500 µL of distilled water was added and incubated for 10 minutes. After that, absorbance was read at 520 nm using spectrophotometer against the blank containing water instead of extract. Concentration of flavonoids of the extracts was calculated using the standard curve of catechin which ranged from 100-1000 µg/mL and the results were given in terms of mg/mL of extract.

By applying the method of Lukowski et al. (2022), total terpenoid content was estimated. Each extract (200 µL) was mixed with 1500 µL of chloroform and 200 µL of concentrated sulphuric acid. The mixture was incubated for 30 minutes and the upper layer was removed, and lower layer was dissolved in 1.5 mL of methanol. Absorbance was read at 540 nm against the blank containing water instead of extract. The standard curve of linalool ranging from 100-1000 µg/mL was used and the concentration of terpenoids was calculated.

Based on the method of Saptarini et al. (2016), total phytosterol content was estimated. To 500 µL of each extract, 1500 µL of chloroform was added and the top layer was pipetted out. Then 1500 µL of the ferric chloride-acetic acid mixture was added followed by the addition of 1000 µL of concentrated sulphuric acid. Absorbance was measured at 540 nm against the blank containing water instead of extract and concentration was calculated using the standard curve of β-sitosterol.

Total phenol content was quantified using the method of Iqbal et al. (2015). To 100 µL of each extract, 250 µL of Folin-Ciocalteu reagent was added followed by the addition of 1000 µL of 5% sodium carbonate and incubated for 10 minutes. The absorbance was measured at 720 nm using spectrophotometer against the blank containing water instead of extract. The concentration of phenols was calculated with the standard curve of gallic acid ranging from 100-1000 µg/mL.

Total tannin content was determined by employing the method of Rebaya et al. (2015). To 200 µL of each extract, 1200 µL of vanillin (4.0%) and 600 µL of concentrated hydrochloric acid were added and mixed well. The mixture was incubated for 10 minutes and absorbance was read at 520 nm using spectrophotometer against the blank. The concentration was calculated using the standard curve of catechin.

2.5. LC-MS analysis

An UHPLC-QTOF-MS instrument (Waters, XEVO-G2-XS QTOF) with electro spray ion source was utilised to capture high-resolution mass spectra. The system is combined with a Waters Acquity UHPLC which includes an Acquity auto sampler, sample manager, LC pump, column component oven and PDA detector. Chromatographic separation was performed using BEH C18 reverse phase column (2.1 x 50 mm, 1.7 mm particle size). Mobile phase A (HPLC grade water) and solvent B (Acetonitrile with 0.1% formic acid) and a linear gradient elution program was applied as follows: 0-1.0 min (20% A & 80% B); 1.1-2.0 min (40% A & 60% B); 2..1-3.5 min (85% A & 15% B); 3.6-5.0 min (90% A & 10% B); 5.1-7.0 min (50% A & 50% B); 7.1-8.0 (20% A & 80% B) by given total run time of 8.1 min. The flow rate was maintained at 0.3 mL/min and the column temperature was set at 30 °C. Injection volume was set up in 10 µL and the pressure limit was 15,000 psi. Mass determination of phytochemicals from each sample was performed using the following MS conditions. The instrument was operated with ESI source in negative ion mode. The ionization was optimized in -ve mode at source conditions: Capillary voltage 3.0 kV; cone voltage 35 kV; source temperature 120 °C and de-solvation temperature, 350 °C. Nitrogen gas was supplied from an in-house generator and gas flow was set at 50 and 800 L/h for cone and de-solvation, respectively. High-purity argon (99.9%) was used as a collision gas at a flow rate of 1.5 mL/min. The collision energy was set at 6.0 eV and mass-spectra were obtained in the continuum mode across the range from 150 to 1000 *m/z*.

2.6. Column chromatography

The selected methanolic extract based on phytochemical analysis and quantification was subjected to column chromatography. For column chromatography, a stationary phase made up of silica gel (60-120 mesh, Merck, India) was packed with hexane in a glass column (38 cm long x 2.2 cm wide) without the presence of air bubbles. For saturation, 200 mL of hexane was used to wash the column. The silica column was loaded with the dry slurry of methanolic extract of *F. racemosa*. Hexane, ethyl acetate and methanol were used in different combinations (hexane + ethyl acetate and ethyl acetate + methanol) for the elution process. A total of 30 fractions (F) each with 25 mL volume were eluted, including F1-F2 (100% hexane), F3-F9 (50% ethyl acetate in hexane), F10-F16 (100% ethyl acetate), F17-F23 (50% ethyl acetate in methanol) and F24-F30 (100% methanol). The total phenolic concentration (TPC) analysis was performed for all the fractions and the higher TPC containing fraction (F18) was further subjected to column chromatography for eluting the sub-fractions (Supplementary Fig. 4). Different solvent combinations (hexane + ethyl acetate & ethyl acetate + methanol) were used to elute 23 sub-fractions like SF1-SF3 (100% hexane), SF4-SF6 (50% ethyl acetate in hexane), SF7-SF9 (100% ethyl acetate), SF10-SF13 (75% ethyl acetate in methanol), SF14-SF17 (50% ethyl acetate in methanol), SF18-SF20 (25% ethyl acetate in methanol) and SF21-SF23 (100% methanol). All these sub-fractions (SF1-SF23) were analyzed for TPC and the selected fractions SF11, 12 15, 19 & 21 were screened for phytochemicals, of which only SF15 contained

high TPC and devoid of all other phytochemicals (tests as mentioned in subsection 2.3) was selected for characterization through direct MS analysis.

2.7. Direct mass spectrometry

Mass determination of phytochemicals in selected fraction SF15 was performed using the negative ion mode mass spectrometry analysis. The instrument was operated with electron spray ionization (ESI) source in the negative ion mode. The ionization was optimized in negative volts mode at given source conditions (capillary voltage 3.0 kV; cone voltage 35 kV; source temperature 120 °C and de-solvation temperature 350 °C). Nitrogen gas was supplied with the flow set at 50 and 800 L/h for cone and de-solvation, respectively. High-purity argon (99.9%) was used as a collision gas at a flow rate of 1.5 mL/min. The collision energy was set at 6.0 eV and mass spectra were obtained in the continuum mode across the range from 400 to 800 *m/z*.

2.8. Superoxide radical scavenging

Superoxide radical scavenging activity of *Ficus* fraction SF-15 was determined by the method of Zhishen et al. (1999). In the reaction mixture, there were 100 µL of different concentrations (10-0.125 mg/mL) of fraction SF15, 1 mL of riboflavin (0.003 mM), 1 mL of methionine (10 mM) and 1 mL of NBT (0.1 mM), which was held under a fluorescent lamp (15 W) for 15 minutes. A formazan with a purple color was generated because of the reaction between superoxide radicals with NBT. The absorbance of illuminated samples was noted at 560 nm and the percentage inhibition of radical scavenging activity was calculated using the formula:
Radical scavenging activity (%) = ((Control absorbance - test absorbance)/Control absorbance) x 100 (Eqn. 1)
On the basis of the concentration versus scavenging activity curve, the IC_{50} value was determined.

2.9. Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity was measured by following the methodology of Buyukbalci and Nehir (2008). Series of dilutions were prepared for the SF15 fraction (10.125, 0.250, 0.500, 1.00 and 10.0 mg/mL) using methanol. A reaction mixture was prepared by adding 0.1 mL of the SF15 fraction, 0.1 mL of H_2O_2 solution (0.5%), 1 mL of potassium iodide solution (1.0%), 1 mL of HCl (3.0%) and mixed well. This was followed by the addition of 1.5 mL of toluidine blue solution (0.1% *w/v*), 1 mL of sodium acetate and mixed well. Control group was prepared by adding 1 mL of H_2O instead of SF15 fraction sample. Absorbance was measured at 600 nm spectrometrically using spectrophotometer and gallic acid was used as the standard for determining the hydrogen peroxide scavenging activity**.**

2.10. Carbonyl assay

Determination of protein oxidation inhibition was performed by following the procedure of Frijhoff et al. (2015). The total protein-bound carbonyl content was calculated by derivatizing the protein carbonyl adducts with dinitrophenyl hydrazine (DNPH). This results in a stable dinitrophenyl hydrazone product, which was then measured spectrophotometrically using spectrophotometer at 540 nm. The goat skin tissue, which collected from local slauther-house, was homogenized with 10 mL of TCA (10%) and collected in a separating funnel and defatted with 10 mL of hexane. The protein-containing bottom layer was taken for the carbonyl test. Tissue lysate of goat skin (100 µL) was combined with *Ficus* fraction (100 µL), 500 µL of $FeSO_4$ (20 mM) and H_2O_2 (5.0%) and incubated for 1 hour at 37 °C in the dark. Then, 500 µL of the DNPH solution (5 mM in 2.5 M HCl) was added. After centrifuging the mixture at 2000 rpm for 5 minutes, the pellet was collected and extracted four times with 1 mL of a solution of ethyl acetate and ethanol (1:1, *v/v*) to get rid of the DNPH. Following the extraction, the pellet was diluted in 1 mL of guanidine hydrochloride (6 M) in order to measure the absorbance at 540 nm against the blank. Gallic acid was used as standard and the inhibition of protein oxidation level was calculated and expressed on percentage basis.

2.11. TBARS assay

Inhibition of lipid peroxidation in goat skin model was estimated using TBARS assay (Nivedha et al., 2020). The amount of malonaldehyde (MDA), a by-product of fatty acid peroxidation that turns pink when it reacts with 2-thiobarbituric acid (TBA), which was measured to determine the extent of lipid peroxidation. The goat skin tissue (0.5 g) was homogenized with 10 mL of TCA (10.0%) and collected in a separating funnel and 10 mL of hexane was added and mixed well with a closed lid and rested for 5 min. The lipids were separated as top layer (100 µL) and mixed with *Ficus* fraction (100 µL) and combined with 500 µL of $FeSO_4$ (20 mM) and H_2O_2 (5.0%). Then, the mixture was incubated for 1 h at 50 °C and after cooling, 1 mL of trichloroacetic acid, (TCA, 10%) and thiobarbituric acid (TBA, 0.8% at pH 4) were added. The mixture was further incubated at 90 °C for 30 minutes and the absorbance was determined at 520 nm against the blank (without tissue lysate) and standard (with gallic acid instead of the fraction).

2.12. Statistical analysis

All the experiments carried out with five replicates (n = 5). All the data were expressed as mean ± standard deviation and statistical analysis was performed by using Student's t-test using excel programme (MS office) to determine significance difference between the control and treated samples. The asterisk symbol on the bars indicate that the values are significantly different (*p < 0.05) when compared to untreated control.

3. Results and Discussion

3.1. Qualitative results of phytochemicals

There are different class of phytochemicals such as alkaloids, flavanoids, terpenoids, phytosterols,

saponins, phenols and tannins present in *Ficus racemosa* bark extracts (Supplementary Table 1). Among these photochemicals, alkaloids generally have tertiary amine, which reacts with Dragendroff's reagent (potassium bismuth iodide) formed an orange-red complex in only water extract of *F. racemosa* with low intensity. Flavonoids react with sodium hydroxide and gave yellow-colored compound in methanolic extract with high-intensity, low intensity in water extract and absent in other extracts (Supplementary Table 1). Terpenoids react with concentrated sulphuric acid and formed carbocation which imparts dark red color in all extracts of *F. racemosa* with high intensity in methanolic extract. Phytosterols react with chloroform, acetic acid, and concentrated sulphuric acid and formed a red colored bi-sterol complex in the lower layer in all extracts of *F. racemosa* with high intensity in methanolic extract and medium intensity in water extract. Saponins did not react with distilled water to align themselves vertically on the surface with a hydrophobic end oriented away from the water and does not results in prolonged foam showing the absence in all the extract of *F. racemosa* bark (Supplementary Table 1). Phenols were present with high intensity in methanolic extract and medium intensity in water extract, which react with ferric chloride to form ferric phenoxide which gaves dark blue color. Tannins on reaction with lead acetate formed creamy precipitate (lead tannates) in methanolic extract of *F. racemosa* with high intensity, low intensity in water extract and absent in other extracts. Thus, the overall qualitative analysis shows methanolic extract has different class of phytochemicals like flavonoids, terpenoids, phytosterols, phenols and tannins in high amounts. In agreement to our results, a previous literature revealed that ethanolic extract of *F. racemosa* bark had the presence of flavanoids, terpenoids, phytosterols, phenols, tannins and absence of alkaloids and saponins (Veerapur et al., 2009).

3.2. Quantitative results of phytochemicals

The total phenols in the *F. racemosa* extract react with phosphomolybdic-phosphotungstic acid of Folin-Ciocalteu reagent and gave blue chromophore in alkaline medium (Blainski et al., 2013). Methanolic extract of *F. racemosa* bark has significantly ($p < 0.05$) higher concentration of phenols (29.93 mg/mL) than the other extracts (Fig. 1A). Phenols have antioxidant property (Kumar and Goel, 2019). Similar to our results, a previous report showed that the total phenols content was high in *F. racemosa* (12.36 mg GAE/100 g) (Sulaiman and Balachandran, 2012). Tannin concentration was significantly ($p < 0.05$) higher in methanolic extract of *F. racemosa* (8.98 mg/mL) compared to other extracts (Fig. 1A). The reaction was binding of aldehyde group of vanillin to sixth carbon of tannins present in the extract to form red chromophore (Schofield et al., 2001). Pharmacological activities of tannins include antioxidant (Skrovankova et al., 2015) and anti-microbial (Marin et al., 2015). Ellagic acid is a tannin compound isolated from *F. racemose* bark (Ahmed and Urooj, 2010b).

The extract containing flavonoids with the C-4 keto group and C-3 or C-5 hydroxyl group reacts with aluminium chloride in an alkaline medium and forms acid-stable reddish color complexes. High amount of flavonoids 0.23 ± 0.08 mg/mL was present in chloroform extract of *F. racemosa* bark extract followed by water extract (0.20 ± 0.04 mg/mL) (Fig. 1B). Pharmacological properties of flavonoids includes anticancer, antioxidant, anti-inflammatory and antiviral properties (Ullah et al., 2020). Bergenin (flavonoid) was the major flavonoid component reported in *F. racemosa* bark (Yadav et al., 2015). The alkaloids in the extract was precipitated as alkoloid-bismuth iodide complex when Dragendroff's reagent was added and in the presence of thiourea, it produces the yellow colur (Sreevidya and Mehrotra, 2003). The water extract of *F. racemosa* bark was quantified to have a significantly ($p < 0.05$) higher alkaloid concentration of 0.18 ± 0.01 mg/mL than the other extracts (Fig. 1B). Alkaloids have antioxidant, anti-inflammatory and anti-cancer activities (Heinrich et al., 2021). No alkaloidal compound reported previously in *F. racemosa* bark.

Terpenoids in the extract react with chloroform to give derivatives of alcohol, which reacts with concentrated sulphuric acid to give a reddish-brown complex (Das et al., 2014). The maximum concentration of terpenoids (0.73 ± 0.01 mg/mL) was found in methanolic extract of *F. racemosa* bark followed by water extract (0.27 ± 0.01 mg/mL) and chloroform extract (0.24 ± 0.01 mg/mL) (Fig. 1C). Wound healing (James and Dubery, 2009) and anti-inflammatory (Vasas and Hohmann, 2014) properties are known for terpenoids. Lupenol is a terpenoid compound present in *F. racemosa* bark (Shiksharthi and Mittal, 2011). Significantly ($p < 0.05$) maximum phytosterol content of 3.28 ± 0.03 mg/mL was present in methanolic extract of *F. racemosa* bark extract, which is followed by water extract (1.23 ± 0.02 mg/mL) (Fig. 1C). Phytosterol in the extract reacts with ferric-chloride-acetic acid and concentrated sulphuric acid mixture to gives a pinkish red color complex (Saptarini et al., 2016). Anticancer (Ali et al., 2015), anti-inflammatory (Kariuki et al., 2012) and anti-diabetic (Nualkaew et al., 2015) are the pharmacological activities reported for phytosterols. Beta-sitosterol and stigmasterol were isolated from *F. racemosa* bark (Shiksharthi and Mittal, 2011).

This quantitative analysis shows that methanolic extract of *F. racemosa* bark has high content of flavonoids, terpenoids, phytosterols, phenols and tannins and hence, it was chosen for further investigation. Traditionally, water extract of *F. racemosa* bark has been used for several medicinal purposes. In this study, the qualitative and quantitative analysis provides that methanolic extract had significantly higher content of phytochemicals than water extract. Therefore, methanolic extract of *F. racemosa* bark can be used for medicinal uses for higher efficiency.

3.3. Comparison of different *Ficus* species barks

The phytochemical screening of methanolic extract of bark material of different *Ficus* species such as *Ficus racemosa*, *F. religiosa*, *F. benghalensis* and *F. hispida* were compared and the results are shown in Fig. 2 and Supplementary Table 2.

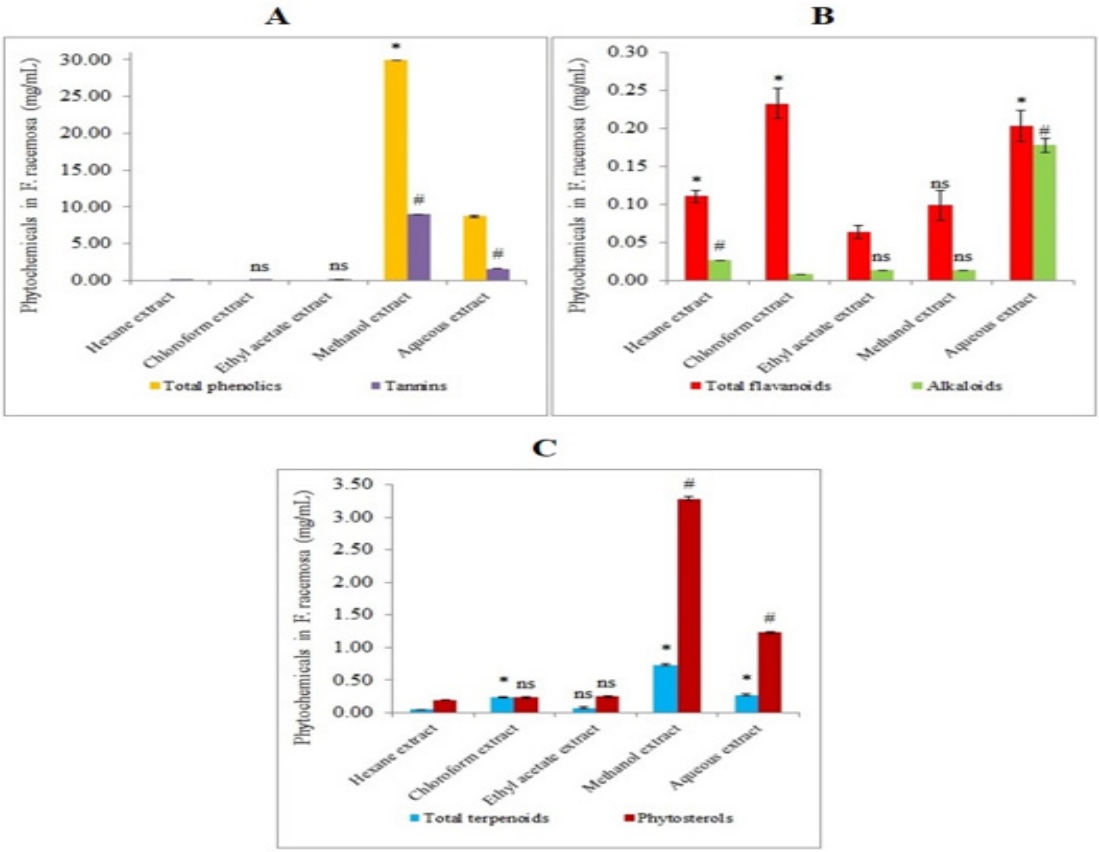

Fig. 1. Quantitative results on various phytochemicals including total phenols and tannins (**A**), flavonoids and alkaloids (**B**) and terpenoids & phytosterols (**C**) in different solvent extracts of *Ficus racemosa* bark. According to Student's t-test, * symbol on each bar indicates statistical significance ($p < 0.05$) of phytochemical content in each extract compared to the respective phytochemical content of hexane extract. The symbol 'ns' on the bar indicates that the value is not significant compared to the hexane extract.

F. racemosa contains significantly ($p < 0.05$) higher level of total phenolic compounds (29.93 mg/mL), tannins (8.98 mg/mL) and phytosterols (3.28 mg/mL) when compared to other *Ficus* species (1.57-19.06 mg/mL of total phenols; 0.30-5.57 mg/mL of tannins and 0.29-1.15 mg/mL of phytosterols). On the other hand, flavonoid level is significantly lower in *F. racemosa* (0.10 mg/mL) when compared to other *Ficus* species (4.10-8.95 mg/mL) (Fig. 2A). *F. religiosa* (0.24 ± 0.01 mg/mL) and *F. benghalensis* (0.07 ± 0.01 mg/mL) possess significantly ($p < 0.05$) higher level of alkaloids in methanolic extract while it is absent in *F. racemosa* (Fig. 2B). The previous studies also suggested that different class of phytochemicals show variation among species (*F. religiosa*, *F. glomerata*, *F. retusa* and *F. carica*) and total phenols and tannin content was found maximum in *F. religiosa* (Rawat et al., 2012). In another study, *Pinus sylvestris* had rich polyphenols and tannins while terpenoids are more in the related species *Pinus nigra* (Nisca et al., 2021). Among the different species, *F. racemosa* has high amount of different classes of phytochemicals and if other *Ficus* species barks are used instead of *F. racemosa* due to increased market demand, the medicinal efficiency could change because of the fact that the amount of the phytochemicals in other *Ficus* species is lesser compared to the *F. racemosa* bark. Also, the phytochemical variability detected in the present work among *Ficus* species would be helpful to identify the *F. racemosa* bark and also to discriminate other *Ficus* bark materials.

3.4. Comparison of trunk and branch barks

The phytochemical profile of methanolic extract of *F. racemosa* trunk bark was compared with branch bark in Fig. 3A-B and Supplementary Table 3. Alkaloids and flavonoids content are comparable between branch bark and trunk bark of *F. racemosa*. However, phytosterols (0.20 mg/mL), phenols (0.90 mg/mL) and tannins (0.42 mg/mL) are present significantly ($p < 0.05$) lower amounts in branch bark compared to trunk bark. This shows that trunk bark of *F. racemosa* has high amount of phytochemical than branch bark. The trunk bark of *F. racemosa* is the authentic material that is being used as a drug in Siddha and Ayurveda systems of Indian medicine. Adulteration may happen with branch bark due to increase in market demand for *F. racemosa* trunk bark, which may leads to lower

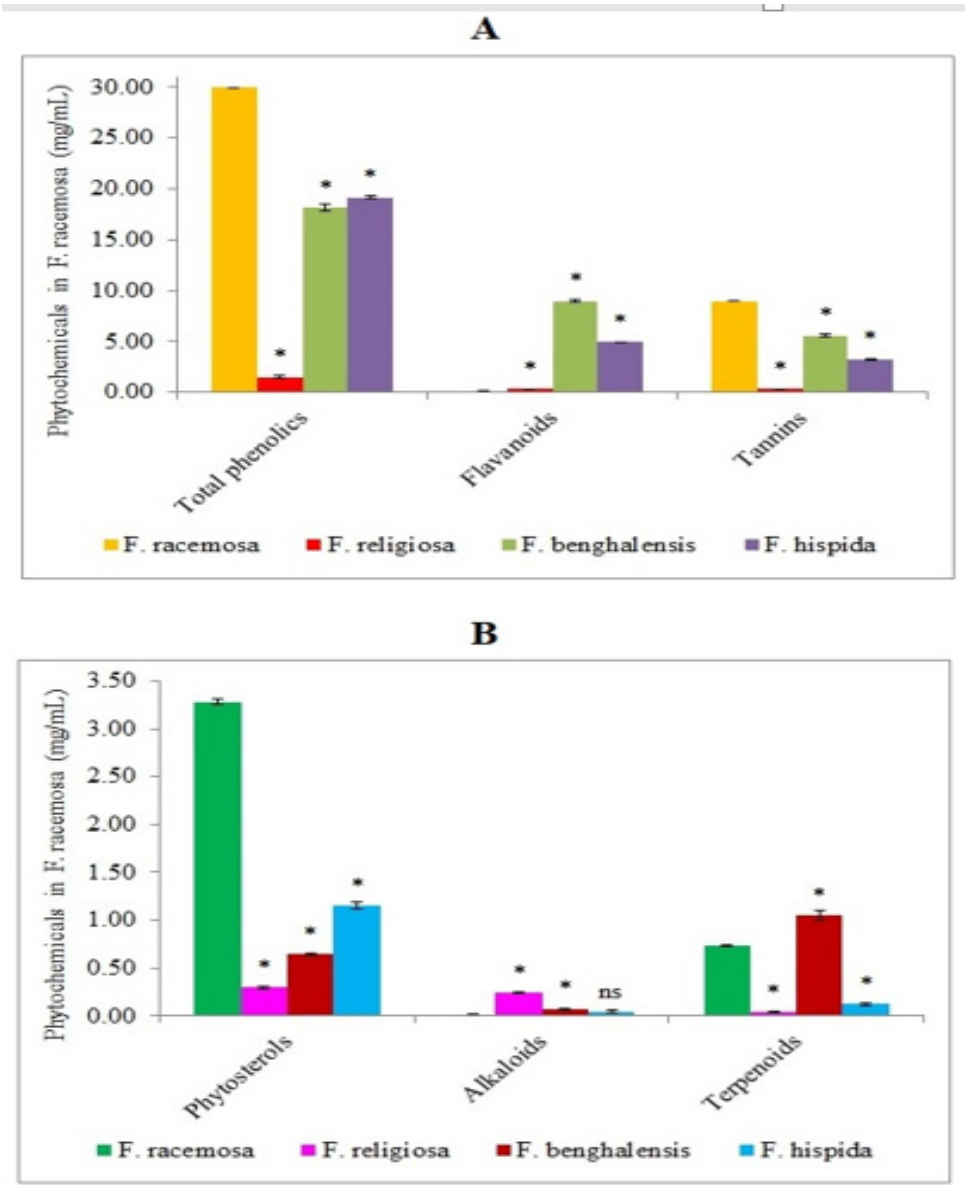

Fig. 2. Variation in the content of phytochemicals including total phenols, flavonoids & tannins (**A**) and phytosterols, alkaloids & terpenoids (**B**) in methanolic extract of bark material of *F. racemosa* and other *Ficus* species. According to Student's t-test, * symbol on each bar indicates statistical significance ($p < 0.05$) of phytochemical content of other *Ficus* species bark compared to the bark sample of *F. racemosa*. The symbol 'ns' on the bar indicates that the value is not significant compared to the *F. racemosa* bark.

the medicinal efficiency. Similar to our results, the phenolic content was reported as higher in trunk bark compared to branch bark in Silver fir (Vek et al., 2022) and Srivastava et al. (2016) also depicted that content of total phenols and flavonoids were lesser in branch bark than trunk bark of *Myrica esculenta*.

3.4. Comparison of trunk and branch barks

The phytochemical profile of methanolic extract of *F. racemosa* trunk bark was compared with branch bark in Fig. 3A-B and Supplementary Table 3. Alkaloids and flavonoids content are comparable between branch bark and trunk bark of *F. racemosa*. However, phytosterols (0.20 mg/mL), phenols (0.90 mg/mL) and tannins (0.42 mg/mL) are present significantly ($p < 0.05$) lower amounts in branch bark compared to trunk bark. This shows that trunk bark of *F. racemosa* has high amount of phytochemical than branch bark. The trunk bark of *F. racemosa* is the authentic material that is being used as a drug in Siddha and Ayurveda systems of Indian medicine. Adulteration may happen with branch bark due to increase in market demand for *F. racemosa* trunk bark, which may leads to lower the medicinal efficiency. Similar to our results, the phenolic content was reported as higher in trunk bark

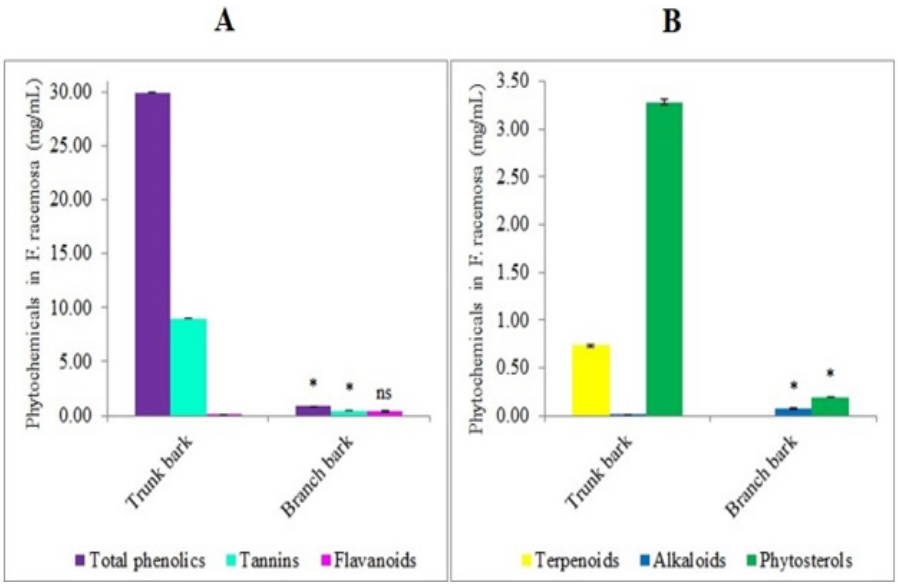

Fig. 3. Comparison of phytochemicals including total phenols, tannins & flavonoids (**A**) and terpenoids, alkaloids & phytosterols (**B**) between methanolic extracts of trunk and branch barks of *F. racemosa*. According to Student's t-test, * symbol on each bar indicates statistical significance ($p < 0.05$) of phytochemical content of branch bark compared to the trunk bark of *F. racemosa*. The symbol 'ns' on the bar indicates that the value of branch bark is not significant compared to the trunk bark of *F. racemosa*.

compared to branch bark in Silver fir (Vek et al., 2022) and Srivastava et al. (2016) also depicted that content of total phenols and flavonoids were lesser in branch bark than trunk bark of *Myrica esculenta*.

3.5. Comparison of bark and wood

Methanolic extract of bark of *F. racemosa* was compared with wood, in which except flavonoids (0.49 ± 0.04 mg/mL), all other phytochemicals like alkaloids (0.06 ± 0.01 mg/mL), terpenoids (0.11 ± 0.01 mg/mL), phytosterols (0.09 mg/mL), phenols (1.01 ± 0.05 mg/mL) and tannins (0.32 ± 0.03 mg/mL) are present in significantly ($p < 0.05$) lower amount in wood compared to *F. racemosa* bark (Fig. 4A-B and Supplementary Table 4). Based on qualitative analysis, saponins was found absent in both bark and wood of *F. racemosa*. This evaluation depicts that wood has very low amount of phytochemicals compared to trunk bark of *F. racemosa*. In agreement to our findings, previous study revealed the presence of more number of phytochemicals (32 compounds) in the bark compared to wood (28 compounds) of *Sonneratia caseolaaris* (Ghalib et al., 2011). This gives clear statement that wood can not be used as an alternative for *F. racemosa* bark even if market demand rises and if used, the therapeutic efficacy could be reduced.

3.6. Effect of storage of bark material

The methanolic extract of freash bark of *F. racemosa* was compared with stored bark, in which all classes of phytochemicals such as flavonoids (4.10 ± 0.04 mg/mL), terpenoids (0.72 ± 0.01 mg/mL), phytosterols (3.76 ± 0.03 mg/mL), phenols (9.87 ± 0.08 mg/mL) and tannins (2.29 ± 0.03 mg/mL) were recorded in stored bark (Fig. 5A-B and Supplementary Table 5). Both phenols and tannin levels of stored bark are significantly ($p < 0.05$) reduced when compared to fresh bark. Alkaloids and saponins are absent in stored bark of *F. racemosa*. Significant increase in phytosterols (3.76 ± 0.03 mg/mL) and non-significant increase in flavonoid contents (4.10 ± 0.04 mg/mL) were noticed during storage compared to fresh bark (3.23 ± 0.03 mg/mL of phytosterols and 0.10 ± 0.02 mg/mL of flavonoids). This shows that the storage for 1 month can change in the amount of phytochemicals in bark of *F. racemosa* and hence it is advisable to use fresh bark of *F. racemosa* to obtain maximal medicinal benefits. Literature also sugested that *Chichona* bark stored for long period results in decreased alkaloid content (Canales et al., 2020).

3.7. UPLC-QTOF-MS results

UPLC-QTOF-MS results for methanolic bark extracts of *F. racemosa*, *F. religiosa*, *F. benghalensis* and *F. hispida* are given in the Fig. 6 and Supplementary Table 6. Out of eight compounds detected, four are present in methanolic bark extract of *F. racemosa*, which includes kaempferol-3-*O*-beta-D-6-malonyl-glucoside, *iso*-orientin, sinomenine and procyanidin C1. The compound kaempferol-3-*O*-beta-D-6-malonyl-glucoside was detected as a first peak at a retention

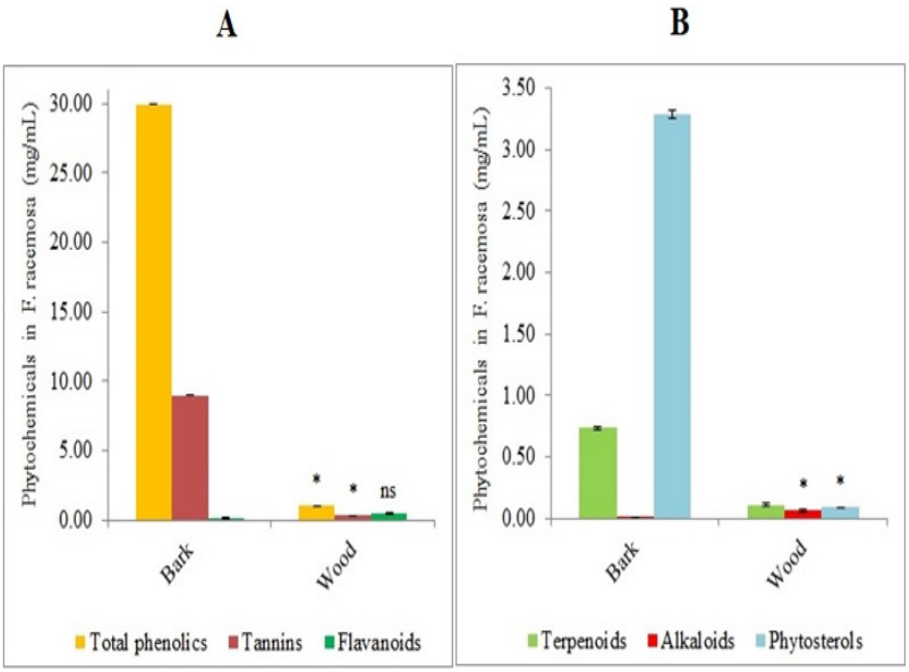

Fig. 4. Differences in the phytochemicals including total phenols, tannins & flavonoids (**A**) and terpenoids, alkaloids & phytosterols (**B**) content of methanolic extract of bark and wood materials of *F. racemosa*. According to Student's t-test, * symbol on each bar indicates statistical significance ($p < 0.05$) of phytochemicals content of wood compared to the trunk bark of *F. racemosa*. The symbol 'ns' on the bar indicates that the value of wood extract is not significant compared to bark extracts of *F. racemosa*.

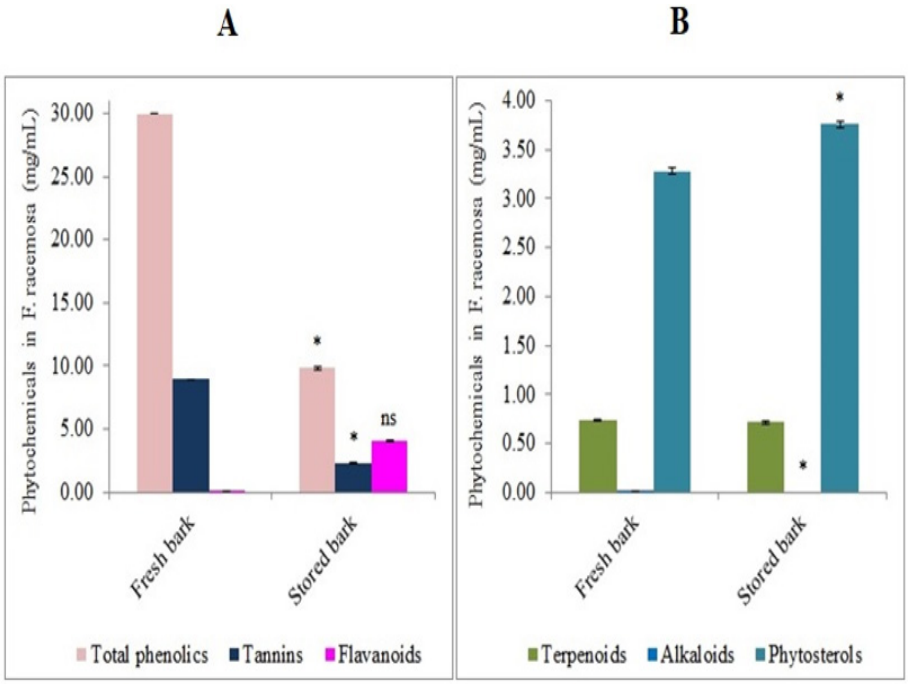

Fig. 5. Variability in the phytochemicals including total phenols, tannins & flavonoids (**A**) and terpenoids, alkaloids & phytosterols (**B**) contents of methanolic extract of fresh and stored bark samples of *F. racemosa*. According to Student's t-test, * symbol on each bar indicates statistical significance ($p < 0.05$) of phytochemicals content of stored bark sample compared to fresh bark of *F. racemosa*. The symbol 'ns' on the bar indicates that the value of stored sample is not significant compared to fresh bark of *F. racemosa*.

time of 1.223 min with parent mass of 533 *m/z* and daughter ions mass of 341 and 191 *m/z*. As the fifth peak, the *iso*-orientin compound was detected at the retention time of 4.450 min and the mass of the parent ion 447 *m/z*. The sinomenine was detected as an seventh peak at a retention time of 4.707 min with the mass of 328 *m/z* parent ion and daughter ion mass of 327 *m/z*. The procyanidin C1 was detected as the eighth peak at the retention time of 4.960 min with 865 *m/z* parent mass and 417 and 289 *m/z* daughter ions mass. Kaempferol-3-*O*-β-D-glucoside (also known as Astragalin) is a flavonoid that had anti-inflammatory and antioxidant properties (Soromou et al., 2012). Iso-orientin is a *C*-glucosyl flavone with robust antioxidant, anti-inflammatory and ameliorative properties against hyperglycemia, hyperlipidemia and insulin resistance (Ziqubu et al., 2020). Sinomenine is an alkaloid derived compound, which has anti-inflammatory effects, analgesic properties and antioxidant properties (Jiang et al., 2020). Procyanidin C1 is a flavonoid, having potent pharmacological properties, such as antioxidant, antibacterial, anti-inflammatory and anti-tumor activities (Chen et al., 2022).

Meranzin is the only compound in methanolic bark extract of *F. religiosa* detected at retention time of 5.453 min and parent mass was 260 *m/z*. It has the ability to reverses psychosocial stress-induced mood disorders, gastrointestinal dysfunction and cardiac disease (Liu et al., 2021). In methanolic bark extract of *F. benghalensis* there are two compounds quinic acid and loganic acid. Quinic acid was detected at retention time of 1.240 min with parent mass 191 *m/z*. Radioprotection, anti-neuroinflammatory and antioxidant are pharmacological activities of quinic acid (Jang et al., 2017). At the retention time of 4.977 min, loganic acid was detected with parent mass 375 *m/z* and daughter ions mass of 353, 351, 215 *m/z*. Methanolic bark extract of *F. hispida* was found to have one compound, oleanane. Oleanane was detected at retention time of 1.223 min with parent mass 440 *m/z* and daughter ions mass of 341, 197 and 195 *m/z*. It suppresses inflammation and oxidative stress (Liby et al., 2012).

The comparison among the UPLC-QTOF-MS results for methanolic bark extracts of *F. racemosa*, *F. religiosa*, *F. benghalensis* and *F. hispida* was found to have no similarity in matched compounds. This shows that there were difference in phytpchemical compounds present in different species belonging to same genus. Also, it highlights that if other *Ficus* species are used in place of *F. racemosa* the efficacy may decrease due to difference in compound. This is supported by the literature (Singh et al., 2016), in which bark extract of six *Terminalia* species was found to have different compounds. The variation in the phytochemical profile registered in UPLC-QTOF-MS study could be employed as species-specific chemical markers to identify and authenticate actual genuine herbal material (*F. racemosa*) and also to discriminate the other morphologically similar *Ficus* species bark samples.

3.8. Purification of *F. racemosa* extract

There were 30 fractions collected from column chromatographic separation of methanolic extract of *F. racemosa* bark. Among the 30 fractions F18 fraction had high total phenolic content of 14.29 mg/mL (Supplementary Fig. 4), which was subjected for further purification by column chromatography and 23 sub-fractions were collected. Among the collected sub-fractions, SF11, SF12, SF15, SF19 and SF21 showed higher TPC of 4.82, 2.97, 4.04, 3.14 and 3.48 mg/mL, respectively. Among these sub-fractions, SF11 and SF12 had other phytochemicals like terpenoids and phytosterols in addition to phenols and tannins, so they were considered mixture of several compounds and hence not selected for further analysis (Supplementary Table 6). In SF15, 19 and 21, only phenols and tannins were detected and based on TPC value, SF15 was selected for direct MS analysis and also subjected to *in vitro* antioxidant assays. Direct MS (negative ion mode) result of fraction SF15 was given in Supplementary Fig. 5. In direct MS, the X-axis represented in mass to charge ratio (*m/z*) and Y-axis in percentage of mass signal abundance (%). The mass peak signals detected at 533, 341 and 191 *m/z* were matched with mass bank library and identified as kaempferol-3-*O*-β-D-glucoside. Medicinal value of this compound includes anti-microbial effects (Rigano et al., 2007), neuroprotective effects (Kim et al., 2017) and cardioprotective effect (Zhou et al., 2019). Hence, presence of kaempferol-3-*O*-β-D-glucoside might play a vital role in the medicinal properties exhibited by *F. racemosa* bark.

3.9. Superoxide scavenging activity

Superoxide is a reactive oxygen species (ROS) containing a negatively charged dioxygen group. Its dynamic changes can provide broad implications in physiological and pathological conditions. Superoxide anion is produced from molecular oxygen by single electron addition and it serves as a precursor for free radicals such as hydrogen peroxide, hydroxyl and nitric oxide (Costa et al., 2021). Both superoxide and its daughter radicals have the potential to react with biological macromolecules and thereby induce tissue damages. In this assay, photo-induced reduction of riboflavin leads to superoxide radical formation from surrounding oxygen in presence of methionine and the generated superoxide radical, which reduces the NBT into purple color formazan that can be measured at 560 nm (Liang et al., 2015). Superoxide radical was found to be scavenged moderately (55.56 ± 3.14% at 10 mg/mL) by fraction SF15 with IC_{50} value of 0.92 mg/mL, which was significantly ($p < 0.05$) lower than that of gallic acid (75 ± 2.07% at 10 mg/mL, IC_{50} = 0.62 mg/mL) (Fig. 7A). However, the *Ficus* fraction exhibits comparable scavenging activity to that of gallic acid at low concentrations of 1 mg/mL (54.44 ± 1.57%), 0.250 mg/mL (35.56 ± 3.14%) and 0.125 mg/mL (22.22 ± 3.14%). The scavenging activity can be increased by increasing the concentration of fraction of *F. racemosa* bark extract. Such superoxide scavenging can reduces oxidative stress in cells and thereby promoting overall cellular health (Lim et al., 2021).

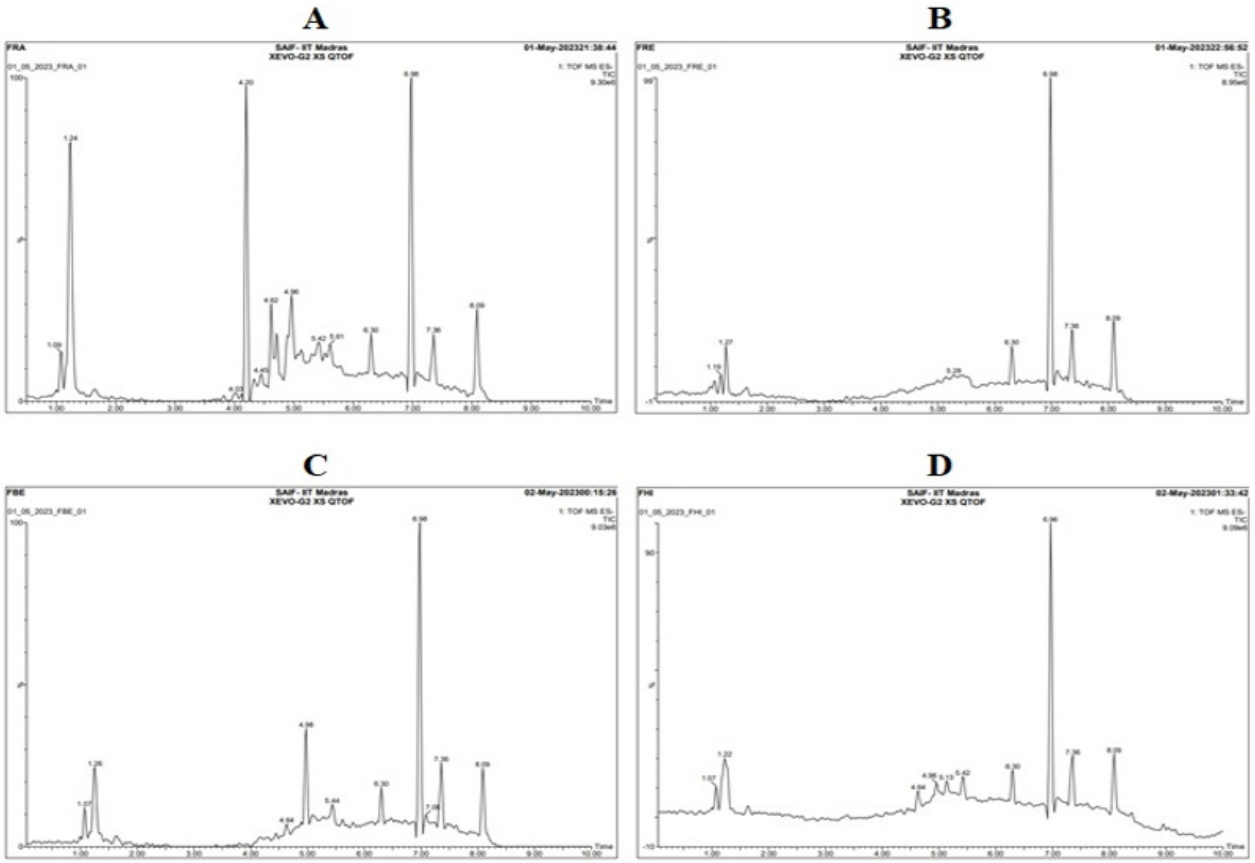

Fig. 6. Phytochemical profile of *Ficus racemosa* (**A**), *F. religiosa* (**B**), *F. benghalensis* (**C**) and *F. hispida* (**D**) analyzed through UPLC-QTOF-MS technique.

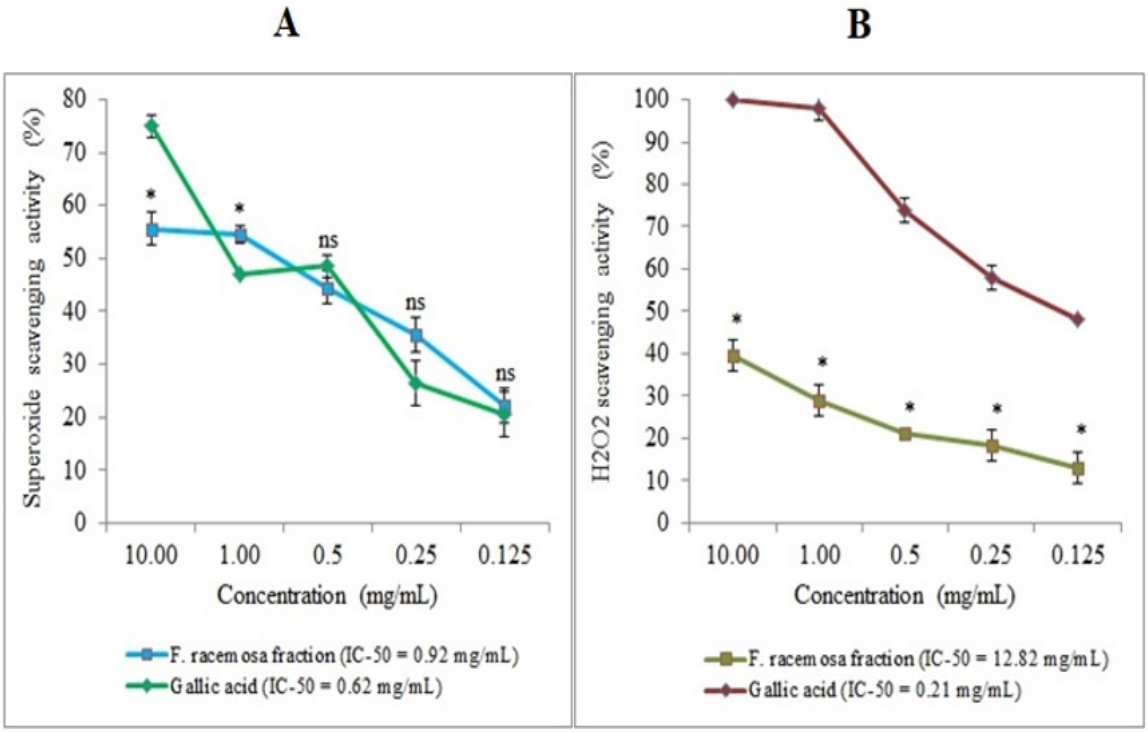

Fig. 7. Free radical scavenging activity of *F. racemosa* fraction against superoxide (**A**) and hydrogen peroxide radicals (**B**). According to Student's t-test, * symbol on each point indicates statistical significance ($p < 0.05$) of *F. racemosa* fraction compared to the standard gallic acid. The symbol 'ns' on each point indicates that *F. racemosa* fraction is not significant compared to gallic acid.

3.10. Scavenging effect on hydrogen peroxide

Hydrogen peroxide is produced when oxygen reacts with certain metal ions, such as iron or copper, or with enzymes called peroxidases. Hydrogen peroxide is highly reactive and damages various biomolecules in cells, including proteins, lipids and DNA, leads to oxidative stress. Hydrogen peroxide scavenging activity refers to the ability of certain substances or compounds to remove hydrogen peroxide from biological systems and prevent its harmful effects (Phaniendra et al., 2015). In this experiment, hydrogen peroxide reacts with potassium iodide in acid medium liberating iodine. The liberated iodine bleaches the blue color of toluidine blue in the presence of sodium acetate to a colorless species and is measured at 600 nm (Pasha et al., 2016). The hydrogen peroxide scavenging activity of fraction SF15 of *F. racemosa* bark extract was significantly ($p < 0.05$) less effective in all the concentrations (39.47 ± 3.72% at 10 mg/mL) with IC_{50} value 12.82 mg/mL compared to the standard gallic acid (100% at 10 mg/mL) which has IC_{50} value of 0.21 mg/mL (Fig. 7B). Generally hydrogen peroxide is involved in the development and progression of cancer by promoting DNA damage and cellular mutations (Vilema-Enríquez et al., 2016). Scavenging hydrogen peroxide may help to prevent the development of diseases like cancer. Also, excessive amounts of hydrogen peroxide can delay wound healing and hence scavenging excess hydrogen peroxide can promote faster healing of wounds (Loo et al., 2012).

3.11. Inhibition of lipid peroxidation

Free radicals like superoxide, hydrogen peroxide and hydroxyl radicals attack lipids that have carbon-carbon double bonds during a process known as lipid peroxidation, which worsens the integrity of biological membranes (Ayala et al., 2014). Lipid peroxidation refers to the oxidative degradation of lipids, particularly unsaturated fatty acids by ROS. Goat skin's fatty material was added to the sample in this case along with ferrous and hydrogen peroxide (Fenton reaction). As a result, aldehydes are produced, malondialdehyde (MDA) being one of them, which is a significant indicator of oxidative damage. The TBA assay (Thiobarbituric acid reactive substances, TBARS) is a commonly used method to measure the levels of lipid peroxidation in biological samples (Khoubnasabjafari et al., 2015). The TBARS assay is based on the reaction between malondialdehyde (MDA, a byproduct of lipid peroxidation), and TBA, which forms a pink-colored complex that can be measured spectrophotometrically. The lipid peroxidation inhibition capacity of fraction SF15 was 74.44 ± 1.57% which is nearly same (not significant) as standard gallic acid (81.11 ± 2.71%) at 10 mg/mL concentration (Fig. 8A). The uses of inhibition of lipid peroxidation include protection against oxidative stress, inflammation, aging and prevention of chronic diseases.

3.12. Inhibition of protein oxidation

Protein oxidation is the process through which ROS including superoxide, hydrogen peroxide and hydroxyl radicals can alter the proteins. Numerous oxidative modifications, such as carbonylation, sulfonation, and nitration may arise as a result of protein oxidation (Gonos et al., 2018). The carbonyl test is a widely used method to measure the levels of protein carbonylation, which is a marker of oxidative damage to proteins (Song et al., 2020). It provides valuable information about the

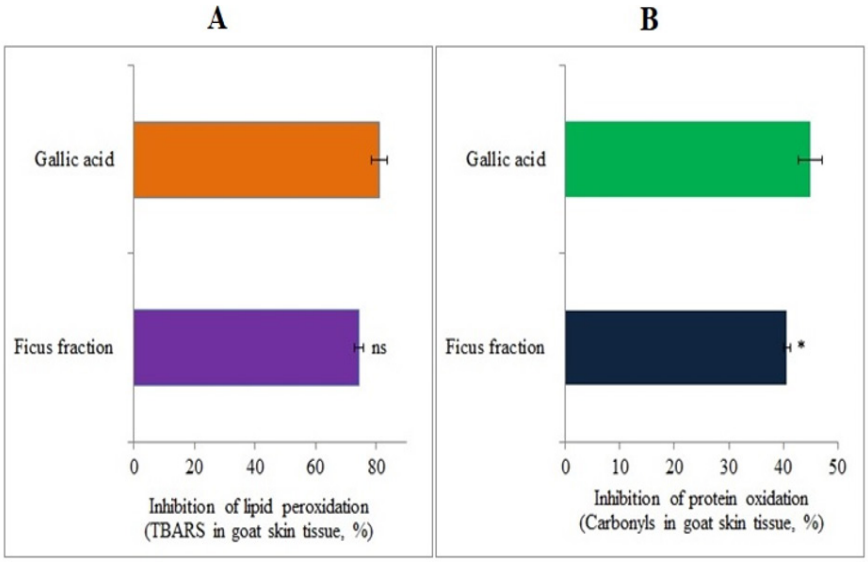

Fig. 8. Inhibition capacity of *F. racemosa* fraction against lipid peroxidation (**A**) and protein oxidation (**B**) in goat skin tissue model. According to Student's t-test, * symbol on each bar indicates statistical significance ($p < 0.05$) of *F. racemosa* fraction-treated sample compared to the standard gallic acid-treated sample. The symbol 'ns' on each point indicates that *F. racemosa* fraction is not significant compared to gallic acid.

efficacy of interventions aimed at preventing or reducing protein oxidation. Protein carbonylation can occur when proteins are exposed to ROS and other oxidative agents, leading to the formation of carbonyl groups on specific amino acid residues. Here, hydrogen peroxide and ferrous are introduced to proteins of goat skin, which oxidized the proteins and led to the formation of carbonyls. The DNPH reacts with the carbonyl group in the proteins to form dinitrophenylhydrazone, which can be measured spectrophotometrically (Apriceno et al., 2018). The protein oxidation inhibition capacity of fraction SF15 (40.65 ± 2.15%) was found significantly ($p < 0.05$) lower than that of standard gallic acid (45 ± 2.12%) (Fig. 8B). It shows that the capacity of *Ficus* fraction SF15 was found moderately effective to inhibit the oxidation of proteins. Inhibition of protein oxidation is an important target to mitigate oxidative stress-related diseases. As protein oxidation is closely associated with the aging process, inhibition of protein oxidation can prevent age-related diseases (Reeg and Grune, 2015).

4. Concluding remarks

The study concludes that the methanolic extract of *F. racemosa* bark contains higher amount of phytochemicals than aqueous extract, so it could be used to replace aqueous extract, which is traditionally used in Siddha and Ayurveda. Among the different *Ficus* species, methanolic extract of *F. racemosa* bark was found to possess higher content of phytochemicals than *F. religiosa*, *F. benghalensis* and *F. hispida* and therefore, usage of other *Ficus* species bark instead of *F. racemosa* bark could reduce the medicinal efficiency. Trunk bark of *F. racemosa* was quantified to have high amount of phytochemicals than branch bark. Also, bark was found to contain higher concentration of different phytochemicals than *F. racemosa* wood. Stored bark of *F. racemosa* was found to exhibit lesser amount of phytochemicals than fresh bark. These data suggests that trunk bark of *F. racemosa*, which was traditionally used in Indian system of medicine has high content of phytochemicals. So, if other materials such as wood, branch bark or stored bark are used in place of trunk bark, the efficacy may be reduced. The UPLC-QTOF-MS data reveals there are no similar compounds among the *Ficus* species and hence genuine herbal drug F. racemosa bark could be precisely identified by using phytochemical markers. Fraction SF15 purified from methanol extract was identified as kaempferol-3-*O*-β-D-glucoside. *In vitro* analysis of SF15 fraction exhibits moderate superoxide and hydrogen peroxide scavenging activities, high inhibition of lipid peroxidation and moderate inhibition of protein oxidation. As the fraction SF15 exhibited good antioxidant property in skin model, it can be used for the manufacturing various cosmetic products, so that it can protect the cells from oxidative damages caused by free radicals and thus prevents skin disease and aging process.

Supplementary file

Supplementary file includes Supplementary Fig. 1-5 and Supplementary Table 1-7.

Author contribution statement

All authors contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript.

Conflict of interest

The authors declare that there is no conflict of interests.

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Original Research Article

Exploration of metabolic variations, anti-cholinesterase, anti-heme biocrystallization, and anti-protein denaturation activities of ten *Capsicum* accessions under different stages of ripening

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ABSTRACT

Hydro-methanolic extracts of pericarps and seeds of 10 different *Capsicum* cultivars from three different species viz., *Capsicum chinense*, *C. annuum* and *C. frutescens* were assessed to determine metabolic variations and anti-cholinesterase, anti-malarial and anti-protein denaturation activities at four different stages of ripening, namely green unripe (GU), turning green-orange (TGO), orange ripe (OR) and red fully ripe (RFR) stages. Many of the ripening stages of the tested cultivars showed significant bioactivities in a dose-dependent manner. In addition, metabolites were identified using GC/MS. On the other hand, HPLC analysis revealed the highest capsaicin and dihydrocapsaicin content in the pericarp of GU, TGO and RFR stages of *C. chinense*. β-Carotene, capsanthin and lutein contents of *C. chinense* were also measured using HPTLC technique. The GU and RFR stages of *C. chinense* contained appropriate amounts of all three carotenoids. These results may be helpful in selecting the cultivars with the best attributes.

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1. Introduction

Plants containing various bioactive ingredients are gaining increasing attention due to their effectiveness in enhancing human health and nutrition (Idris et al., 2017; Unuofin et al., 2017). The proper identification of metabolites with bioactive properties is a fundamental step in any improvement program, such as selecting effective genotypes in plant breeding programs for various pharmacological and nutritional purposes (Chenet al., 2014; Ganie et al., 2015). Medicinal plants have long been revered for their profound therapeutic properties, offering a natural and holistic approach to healing various ailments. Packed with a myriad of bioactive compounds, these plants possess the ability to alleviate symptoms, boost immunity, and promote overall well-being (Agrawal and Jain, 2023; Singh et al., 2023). From traditional herbal remedies to modern pharmacological applications, the therapeutic potential of medicinal plants continues to be explored and harnessed by cultures worldwide. Whether it's the anti-inflammatory properties of turmeric (Choi et al., 2019), the calming effects of chamomile (Devi and Kumar, 2023), or the immune-boosting prowess of *Echinacea* (Chen and Yu, 2016), these botanical wonders serve as a testament to nature's profound healing capabilities. As we delve deeper into the realm of herbal medicine, we uncover a treasure trove of plant-based remedies that offer gentler alternatives to conventional treatments, empowering individuals to take control of their health in a sustainable and harmonious manner (Mohammad hosseini et al., 2019a; Mohammad hosseini et al., 2019b).

Capsicum, the genus known by different names such as chilli, chile pepper, bell pepper, hot pepper, sweet pepper etc., all over the world, belongs to the family Solanaceae. The family Solanaceae, encompassing diverse plants like tomatoes, potatoes, and bell peppers,

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contributes a wealth of culinary delights and medicinal treasures to our lives. *Capsicum* spp. are considered as one of the oldest, popular vegetables and spices cultivated mostly in the tropical and subtropical parts of the world. Chillies are appreciated not only because of their economic importance but also for their rich nutritional values. Chillies are abundantly produced in India and, because of their cheapest price, they are consumed across all over the country (Mehta, 2017). Several *Capsicum* sp. have been domesticated comprising mild and sweet to hot, strongly pungent, flavoured and aromatic ones. The sweet non-pungent peppers are consumed as vegetables, whereas pungent peppers are employed as hot spice. *Capsicum* plants are perennial shrubs consisting of about 27 species, among which 5 species, namely *Capsicum annuum* L., *C. chinense* Jacq., *C. pubescens* R., *C. baccatum* L. and *C. frutescens* L. are broadly cultivated. (Bosland and Votava, 2012).

The *Capsicum* fruit has been traditionally used as a vegetable, natural coloring agent and as a therapeutic medicine. A diverse group of bioactive phytochemical compounds consisting of phenolics, flavonoids, carotenoids, etc. are present in the fruit of *Capsicum* plants (Altemimi et al., 2017). Besides the vast economic importance of the peppers as vegetables and spices, the active principles present in the fruit of *Capsicum* account for its significant contribution in ethnopharmacological use (Palevitch and Craker, 2012). Dietary antioxidants protect us from cancer, diabetes, cardiovascular diseases, etc. Chilli peppers are rich in protective antioxidants like vitamin C, E, β-carotene, etc. (Villa-Rivera and Ochoa-Alejo, 2020). It has also been documented that *Capsicum* species exhibit varying degrees of antimicrobial properties against *Bacillus cereus, Bacillus subtilis, Clostridium sporogenes, Clostridium tetani, Streptococcus pyogenes* (Cichewicz and Thorpe, 1996) and on *Arcobacter butzleri, Arcobacter cryaerophilus, Arcobacter skirrowii, Campylobacter jejuni* and *Helicobacter pylori* (Dogan et al., 2018). Mature green fruits of *C. annuum* var. *acuminatum* showed α-amylase and α-glucosidase inhibitory activities while its fruits, at the premature green stage, inhibited acetylcholinesterase enzyme by the Ellman method (Loizzo et al., 2008). Aqueous extract of green *C. annuum* exhibited a protective effect against ethanol induced hepatotoxicity by acting as an antioxidant agent. It arrested ethanol induced apoptosis and brought down pro-inflammatory cytokine levels (Das et al., 2018). Chilli pepper is known to exhibit hypocholesteremic and hypolipidemic properties. It can be used in the intervention of cardiovascular diseases (Sanati et al., 2018). Furthermore, chilli pepper extracts are reported to have anti-inflammatory and anti-allergic properties (Jolayemi and Ojewole, 2013). Chilli fruits are used as stomachic remedies and represent preventive properties for diseases like rheumatism, arthritis, heart arrhythmias, bronchitis and chest colds with cough and headache (Pawar et al., 2011). Fruit extract of *C. annuum* and fine powder of *C. chinensis* were reported to have larvicidal activity against *Aedes albopictus* and *Culex quinquefasciatus* (Ombugadu et al., 2020).

Fruit pungency is the most desirable flavour trait found in chillies. Pungency is mainly because of the presence of capsaicinoids, which is a unique characteristic of chilli fruits. No other genus except *Capsicum* contains capsaicinoids. Piperine ($C_{17}H_{19}NO_3$), present in black pepper, has a similar composition as of capsaicin ($C_{18}H_{27}NO_3$), thereby giving a mildly pungent flavor. Capsaicinoids are amides naturally synthesized by condensation of vanillylamine and different-sized fatty acid chains in the presence of capsaicin synthase enzyme within the placenta of chilli fruits. Capsaicin is the most abundant capsaicinoid followed by dihydrocapsaicin. Other minor capsaicinoids are norcapsaicin, nordihydrocapsaicin, nornordihydrocapsaicin, homocapsaicin and homodihydrocapsaicin (Maokom et al., 2014).

Capsicum fruits being one of the most common spices in the world and having a range of medicinal and nutritional value may be explored for their anti-cholinesterase activity for controlling neuro-degenerative diseases, anti-malarial activity to manage chloroquine resistant malaria and anti-protein denaturation activity for the intervention of arthritic pain.

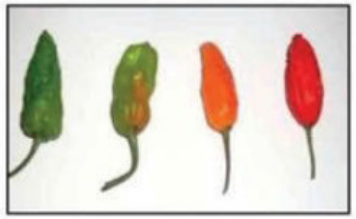
In the present study, we made an effort to understand *in vitro* anti-acetylcholinesterase, anti-malarial and anti-protein denaturation activity of different species and varieties (landraces) of sweet to hot *Capsicum* fruit pericarp as well as seeds along with placenta during their ripening stages from green unripe (GU) to turning green-orange (TGO) to orange ripe (OR) and finally to red fully ripe (RFR) stages. We also intended to identify the compounds present in the hydro-methanolic extracts that can be correlated to the above mentioned bioactivities from the metabolite profiles. Moreover, on the basis of their metabolite profiling using GC/MS based metabolomic and chemometric tools, we discriminated the tested cultivars (landraces) during their ripening stages.

2. Experimental

2.1. Plant materials

The experimental samples were collected from different parts of West Bengal, India based on morphological variations at different stages of ripening from 2016 to 2018 only during the peak growing season, *i.e*, November to December. Ten different types of chilli fruits from three different *Capsicum* species, *C. chinense, C. annuum* and *C. frutescens* were collected (Fig. 1) during the peak winter season, *i.e.*, the months of November to December (Table 1). Fruits were sampled according to different ripening stages namely green unripe (GU), breaker/turning green-orange (TGO), orange ripe (OR) and red fully ripe (RFR) stages. In bird's eye chilli group of *Capsicum frutescens*, only the green black (GB) and black (BL) stages could be collected. In the clustered pendent downward chilli group of *Capsicum annuum*, the ripening stages were green unripe (GU), breaker/turning green-black (TGB) and black-orange ripe (BO).

The taxonomic identification of the studied chilli species and cultivars were done by Prof. Dr. Pinaki Acharya, Department of Horticulture, University of Calcutta, West Bengal, India. The voucher specimens of the different



1. *Capsicum chinense*
cv. Ghee smelling group (8– 9 cm. long)

2. *Capsicum frutescens*
cv. Bird's Eye chilli pepper group (4-5 cm. long)

3. *Capsicum frutescens*
cv. Tobasco group (1-2 cm. long)

4. *Capsicum annuum*
cv. clustered pendent downward
group (10 – 11 cm. long)

5. *Capsicum annuum*
cv. cherry group
(1-2 cm. in diameter)

6. *Capsicum annuum*
cv. pendent anaheim group
(8-10 cm. long)

7. *Capsicum annuum*
cv. small hot, pendent solitary
group (3-4 cm. long)

8. *Capsicum annuum* var. *glossum* of Shimlai Mirchi Group – Green,
Red and Yellow bell pepper (10-12 cm long)

9. *Capsicum annuum* of
long waxy group (15–20 cm. long)

Fig. 1. The morphological variations of the experimental samples displayed along with their ripening stages.

Table 1
List of *Capsicum* spp. studied along with their morphological characters.

Sl. No.	Species / Cultivar.	Name of Cultivated Varieties / Landraces	Morphology, size of fruits	Collection spots, districts
1	*Capsicum chinense* Jacq.	Ghee (a kind of butter) smelling variety	Conical/inflated, pointed tipped, wrinkle surfaced, ridged, pericarp 8-9 cm. long	Barrackpore Station market, North 24 parganas Lat 22.759908 Long 88.370349
2	*Capsicum frutescens* L.	Bird's eye chilli pepper group	Black pericarp, pointed, 4-5 cm. long	Contai market, East Midnapore Lat 21.781134 Long 87.7069292
3	*Capsicum frutescens* L.	Tobasco group	Very small sized, pointed, 1-2 cm. long	Field cultivation of Baruipur, South 24 Parganas Lat 22.374367 Long 88.4328829
4	*Capsicum annuum* L.	Clustered pendent downward, long black group	Pointed, slender, long, black, 10-12 cm. long	Vegetable market, Purulia Lat 22.8780863 Long 86.4994078
5	*Capsicum annuum* L.	Cherry group	Roundish, smooth surfaced, 1-2 cm. in diameter	Field cultivation in Baruipur, South 24 Parganas Lat 22.374367 Long 88.4328829
6	*Capsicum annuum* L.	Pendant Anaheim group	Long, slender, tips pointed, 8-10 cm. long	Vegetable market North Kolkata Lat 22.5671008 Long 88.2156787
7	*Capsicum annuum* L.	Small hot pendent solitary group	Tips not pointed, smooth surface, short, 3-4 cm. long	Vegetable market of Jhargram village, West Midnapore Lat 22.454647 Long 86.9938421
8	*Capsicum annuum* L	Large hot pendent solitary group	Tips not pointed, smooth surface, medium sized, 5-7 cm. long	Vegetable market, North Kolkata Lat 22.5671008 Long 88.2156787
9	*Capsicum annuum* L.	Variety glossum Shimlai Mirchi group- green, red and yellow bell pepper	Pericarp fleshy, not pointed smooth surfaced, ridged, 10-12 cm. long	Lakshmikantapur station market, South 24 Parganas Lat 22.110001 Long 88.322828
10	*Capsicum annuum* L.	Long waxy group	Very long sized, pericarp, smooth, thick, fleshy, not pointed, 15-20 cm. long	Field cultivation in Lauhati, North 24 Parganas Lat 22.608800 Long 88.519092

plant samples were deposited at the Calcutta University Herbarium (CUH), University of Calcutta, Kolkata, West Bengal, India.

2.2. Chemicals and reagents

Acetylcholinesterase enzyme from *Electrophorus electricus*, hematin porcine, chloroquine di phosphate, *N*- methyl-*N* (trimethylsilyl) trifluroacetamide, methoxyamine hydrochloride, fatty acid methyl esters (FAME) markers, vanillic acid, *O*-acetyl salicylic acid, benzene-1,2,4 triol, capsaicin, dihydrocapsaicin and adonitol were purchased from Sigma-Aldrich, St. Louis, USA. Hydrochloric acid, dimethyl sulphoxide, oleic acid, glacial acetic acid, pyridine, sodium dodecyl sulphate, sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium hydroxide, sodium bicarbonate and sodium acetate were procured from Merck Specialities Pvt. Ltd., Mumbai. Gallic acid, ferulic acid, 4-hydroxy benzoic acid, quinic acid, chlorogenic acid, caffeic acid, bovine serum albumin, acetyl thiocholine iodide and 5,5-dithiobis 2-nitro benzoic acid (Ellman's reagent) were obtained from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. 3,4-Dihydroxy benzoic acid was acquired from Hi Media Laboratories Pvt. Ltd., Mumbai. Galantamine hydrobromide, was acquired from Sun Pharmaceutical Industries, India. Ibuprofen was purchased from Abbott India Limited, Goa. All the organic solvents used were of analytical grade purchased from Merck Pvt. Ltd. For GC/MS analysis,

the solvents used were of HPLC grade purchased from Merck Pvt. Ltd.

2.3. Extraction of plant materials

Hydro-methanolic extracts of fruit pericarps and seeds with placenta were prepared by heating the liquid nitrogen crushed plant materials in 50% methanol at 70 °C, for 3 hours in a boiling water bath under constant stirring. The filtrate for each sample was then evaporated to dryness under reduced temperature and pressure. The crude extract of each sample was preserved at -20 °C for further analysis. The extracts were used to evaluate their anti-cholinesterase, anti-malarial and anti-arthritic activity. With the same solvent extracts, GC-MS, HPLC and HPTLC analyses were also performed under the optimal experimental conditions.

2.4. Assay for acetylcholinesterase inhibition

Acetylcholinesterase (AChE) inhibitory property was measured modifying the previously reported method of Debnath et al. (2021). In this relation, AChE from electric eel was used as enzyme source for AChE assay. The 5,5′-dithiobis (2-nitro benzoic acid) (DTNB) was used as color developer for the measurement of cholinesterase activity. A yellow anion (5-thio-2 nitro benzoate) was formed as a result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylcholine. Different concentrations of hydro-alcoholic extracts of pericarp and seed extract (10 µL) were added to 20 µL of AChE (19.93 unit/mL in phosphate buffer, pH 8) and 1 mL of buffer. The reaction was started by adding 10 µL of 5,5′-dithiobis (2 nitro benzoic acid, 0.5 mM) (DTNB) and 20 µL of acetylthiocholine iodide (0.6 mM) solution. The reaction mixture was incubated at 37 °C for 20 minutes. The optical density was measured spectrophotometrically at 412 nm immediately. The percentage inhibition of AChE activity of the plant extracts was calculated using the following formula:

Inhibition (%) = (A - S)/A × 100 (Eqn. 1)

Where A and S respectively represent the OD value for the control and the test sample. From triplicate test results, the data were represented as mean ± standard deviation (SD) and all the obtained results were analysed by Microsoft Excel, 2007. The value of $p < 0.05$ was considered as significant value of the data. Galantamine hydrobromide was used as positive control.

2.5. Assay for hematin polymerization inhibition

The potential of anti-malarial activity was evaluated using the protocol followed by Hussain et al. (2013) with slight modifications. Accordingly, 100 µL of plant sample dissolved in 10% DMSO was incubated with 100 µL of hematin (0.003 M, freshly prepared in 0.1 M NaOH), HCl (1 M) and oleic acid. The reaction volume was adjusted by sodium acetate buffer (0.5 M, pH 5). The samples were incubated for 4 hours with gradual shaking in an orbital shaker. After incubation, the samples were centrifuged at a speed of 14000 rpm, at 21 °C for 10 minutes. The hemozoin pellets were separated from the supernatant and re-suspended in 1 mL of NaOH (0.1 M) and incubated again for 1hour at room temperature. The hemozoin content was determined by measuring the absorbance of samples at 400 nm using UV-Visible spectrophotometer. The percentage inhibition of hematin polymerization inhibitory activity of the plant extracts was calculated following the formula:

Inhibition(%) = (AB - AS)/AB (Eqn. 2)

Here, AB represents the OD value of the blank, and AS denotes the OD value of the samples undergoing testing. The inhibition percentage of triplicate test results were represented as mean ± standard deviation (SD) and all these data were plotted by Microsoft Excel, 2007. The value of $p < 0.05$ was considered as significant value of the data. Chloroquine di phosphate was used as positive control.

2.6. Assay for protein denaturation inhibition

In vitro protein denaturation assay was performed by the method of Mizushima and Kobayashi (1968) along with Sakat et al. (2010) with slight modifications. In this regard, the reaction consisted of a mixture with 200 µL of the test solutions and 200 µL of aqueous bovine serum albumin (BSA, 5%). pH of the reaction mixture was adjusted using glacial acetic acid. The reaction tubes were first incubated at 37 °C for 20 min and thereafter were heated at 70 °C for 10 min. After cooling, the relevant turbidity was measured at 660 nm while using sample along with distilled water devoid of BSA, as a blank. The percentage inhibition of protein denaturation was calculated using the formula:

Inhibition(%) = (A - B)/A (Eqn. 3)

In this context, A stands for the absorbance of the control set, while B represents the absorbance of the tested sample. The triplicates of inhibition(%) for each dilution were represented as mean ± standard deviation (SD) and all the obtained data were evaluated by Microsoft Excel, 2007. The value of $p < 0.05$ was considered as significant value of the data. Ibuprofen was used as positive control.

2.7. Chemical profiling of the herbal extracts using GC/MS analysis

Agilent 7890 A GC [software driver version 4.01 (054)] interfaced with 5795 C inert MSD with Triple Axis Detector was employed for GC-MS analysis. HP-5 MS capillary column [Agilent J&W; GC Columns (USA)] of dimensions 30 m × 0.25 mm × 0.25 µm was used in the analytical arrangement. The method of Kind et al. (2009) was followed with a few modification by Das et al. (2016). The oven temperature program for analysis was set as oven ramp 60 °C (initial 1 min hold), to 325 °C with an increasing rate of 10 °C per min. The oven temperature was held for 10 min before cooling down producing a total run time of 37.5 min. The injection temperature was set at 250 °C, the MSD transfer line at 29 °C and the ion source at 230 °C. Helium was used as the carrier gas at a flow rate of 0.723 mL/min (carrier linear velocity of 31.141 cm/s). Adonitol was added to the dried crude extract and was followed by derivatization using methoxyamine hydrochloride

and *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide. FAME markers prepared in chloroform was added to the sample before injection. Derivatized samples were injected via splitless mode on to the column. A solvent delay of 5.90 minute was allowed to check overloading of sample in the column. Chromatographic peaks and mass spectra were recorded. Automated mass spectral deconvolution and identification system (AMDIS) was used to deconvolute and identify metabolites from chromatographic peaks. The fragmentation patterns of the mass spectra as well as retention times (RT) of samples were compared with entries of mass spectra, RT in Agilent GC-MS Metabolomics RTL Library (2008) (Agilent Technologies, USA). Many metabolites were further confirmed by chromatographic data with that of authentic metabolite samples. Normalization of peak areas of different metabolites were done by dividing the peak area by dry weight of crude extract followed by peak area of adonitol as the internal standard. The relative response ratios obtained by this process was further used for data interpretation.

2.8. HPLC analysis

The HPLC analyses were carried out on an Agilent 1260 infinity series HPLC system equipped with a quarternary pump and a diode array detector. The chromatographic conditions include Waters Symmetry C_{18} column [dimension: 150 mm × 4.6 (internal diameter) and 5 µm particle size]. The UV detection wavelength for both capsaicin and dihydrocapsaicin was set at 280 nm (Usman et al., 2014). Mobile phase consisted of a binary mixture of water and methanol which was run in a linear gradient modifying the method reported by Waite and Aubin (2008). Crude dry samples were extracted using methanol (HPLC grade) and centrifuged. Supernatant was then filtered using a 0.2 µm membrane filter and 20 µL of the sample with a range of concentration from 10 to 50 mg/mL was subsequently injected manually into the system at ambient temperature. The linear gradient elution was applied using 100% water (solvent A) and 100% methanol (solvent B) at a constant flow rate of 1.50 mL/min for a total run time of 20 minutes. The gradients were as follows: 40% B, increased to 85% B over 8 min, increased to 99% B over 5 min and then returned to initial ratio (40% B) over 7 min.
Different concentrations ranging between 0.01 to 15 mg/mL of capsaicin and 0.01 to 3 mg/mL of dihydrocapsaicin were prepared and run into the system. The peak areas of the standards were plotted into a calibration curve to find out unknown concentration of capsaicin and dihydrocapsaicin in the samples. The content of capsaicin and dihydrocapsaicin (µg/mg) of the extracts were calculated from the calibration curve. Then, 20 µL of an eluent containing water and methanol in a ratio of 9:1 was injected after every sample set to avoid measurable carryovers. Injection of both standards were made at the beginning of each work module.

2.9. HPTLC analysis

High performance-thin layer chromatography was carried out on pre-coated silica gel 60F_{254} plates of 0.25 mm thickness and the operating software used was WinCATS. Acetone extracts of only *Capsicum chinense* fruit pericarp were analysed for lutein, β-carotene and capsanthin following Hernandez et al. (2012) and Das et al. (2017).

2.10. Statistical analysis

Each experiment was performed in triplicates and percentage inhibition was calculated using the formula mentioned as Eqn. 1, Eqn. 2 and Eqn. 3. Regression equations were prepared from the concentrations of the extracts and percentage inhibition of enzyme activity using Microsoft Excel, 2007. IC_{50} values as a measure of concentration of sample required to inhibit the enzyme activity by 50% were calculated from the regression equations.

2.11. Multivariate data analysis of polar metabolites

Multivariate analysis such as PLS-DA (partial least squares-discriminant analysis) of different varieties of *C. annuum*, *C. chinense* and *C. frutescens* were carried out with the help of Metaboanalyst 4.0, for metabolomic data analysis and interpretation.

3. Results and Discussion

3.1. AChE inhibition

***Capsicum chinense*:** All the pericarp and seed extracts of four ripening stages (GU, TGO, OR and RFR) of the ghee smelling group inhibited the AChE enzyme in a dose-dependent manner. Both pericarp and seed of the TGO stage showed the highest activity with IC_{50} value of 1.593 ± 0.049 and 1.497 ± 0.037 mg/mL, respectively.
***Capsicum frutescens*:** 50% hydro-methanolic extracts of all the pericarp and seed of erect upward bird's eye chilli group showed inhibition against AChE in a dose-dependent manner. All the extracts of the upward tobacco group except seed of TGO stage inhibited the enzyme. Among these two landraces of *C. frutescens*, the highest activity with the lowest IC_{50} value (0.789 ± 0.048 mg/mL) was observed in the pericarp of BL stage of erect upward bird's eye chilli group followed by the pericarp of GB stage of the same (IC_{50} = 0.833 ± 0.041 mg/mL).
Capsicum annuum: Pericarp and seed extracts of all the ripening stages of clustered pendent downward group inhibited the AChE enzyme in a dose-dependent manner. Both pericarp and seed of OR stage of the cherry group did not show any activity. Pericarp of OR stage and seed of RFR stage of the pendent Anaheim group did not inhibit AChE enzyme. Extracts of all the stages of large hot pendent group except the seed of RFR stage showed AChE inhibitory activity. Only the pericarp and seed of GU stage and pericarp of TGO stage of small hot pendent solitary group showed inhibition against AChE enzyme. The seed extract of GU and RFR stage of the long waxy group did not show any inhibition. None of the seed extracts from the green, yellow and red bell pepper inhibited AChE. However, seed extract of the GU stage of the large hot pendent group and pericarp

extract of BO stage of clustered pendent downward group showed the highest inhibition of all the samples tested.

In case of pericarps, the BO ripening stage of the clustered pendent downward group of *Capsicum annuum* showed the highest activity with an IC_{50} value of 0.671 ± 0.007 mg/mL followed by BL and GB stage of erect upward bird's eye chilli group of *Capsicum frutescens* with IC_{50} values of 0.789 ± 0.05 and 0.833 ± 0.036 mg/mL. In case of seeds, the highest inhibition was observed in the GU stage of large hot pendant group followed by the GU stage of clustered pendent group of *Capsicum annuum* with IC_{50} values of 0.627 ± 0.029 and 0.845 ± 0.041 mg/mL. The IC_{50} values of all the tested samples are depicted in the Fig. 2(A).

3.2. Anti-heme biocrystallization assay

Anti-heme biocrystallization assay was carried out for all the *Capsicum* samples and percentage inhibition was noted at 3 mg/mL for the pericarp and seed extracts of all the ripening stages.

Capsicum chinense: Both pericarp and seed of all the ripening stages of ghee smelling group of this species exhibited anti-heme biocrystallization activity. The highest activity was shown by the pericarp of TGO stage with 91.25 ± 0.68% inhibition followed by seeds of GU stage with inhibition(%) of 89.93 ± 0.36%.

***Capsicum frutescens*:** Both pericarps and seeds of BL and GB stage of erect bird's eye chilli group showed remarkable activities with 99.25 ± 0.29% (pericarp BL), 99.14 ± 0.37% (seed BL), 97.53 ± 0.11% (pericarp GB) and 90.10 ± 0.34% (seed GB) respectively to inhibit the biocrystallization of hematin. Except the seed of TGO stage (30.18 ± 0.58%), seed (48.0 ± 0.66%) and pericarp (46.91 ± 0.69%) of OR stage and seed of RFR stage (44.54 ± 0.66%) of upward tobasco group all the other stages of extractions showed more than 50% inhibition. Pericarp of RFR stage followed by seeds of GU stage of ripening showed higher activities such as 90.32 ± 0.27% and 73.78 ± 0.5%, respectively.

***Capsicum annuum*:** All the ripening stages of pericarps and seeds of pendent anaheim, large hot pendent, small hot pendent solitary, cherry, clustered pendent downward, long waxy and glossum bell pepper groups showed anti-heam biocrystallization activity more than 50%, except the seeds of OR stage of long waxy group. The pericarp of TGO stage of pendent anaheim group, seed of TGO stage of large hot pendent group, seeds of OR and RFR stages of cherry group, pericarp of TGB stage of clustered pendent downward group, seeds of TGO stage of long waxy group and the pericarp of red stage of glossum bell pepper showed 98.94 ± 0.31%, 97.12 ± 0.20%, 99.0 ± 0.27% and 99.18 ± 0.33%, 99.14 ± 0.43%, 99.79 ± 0.11%, 99.75 ± 0.16% inhibition of polymerization of hematin protein which exhibited strong anti-plasmodial or anti-malarial activity. Out of all these values, the highest inhibition was obtained in the seed extract of TGO stage of long waxy group of chilli, *i.e.*, 99.79 ± 0.11%. The anti-heme biocrystallization activity of all the tested samples are represented in the Fig. 2(B).

3.3. Anti-protein denaturation assay

Pericarp and seed extracts of different ripening stages of all the samples were subjected to anti-protein denaturation assay at a concentration of 292.68 mg/mL. Among all the species and cultivars, the highest inhibition was exhibited by the pericarp of BL stage of erect bird's eye chilli group with 99.23 ± 0.16%.

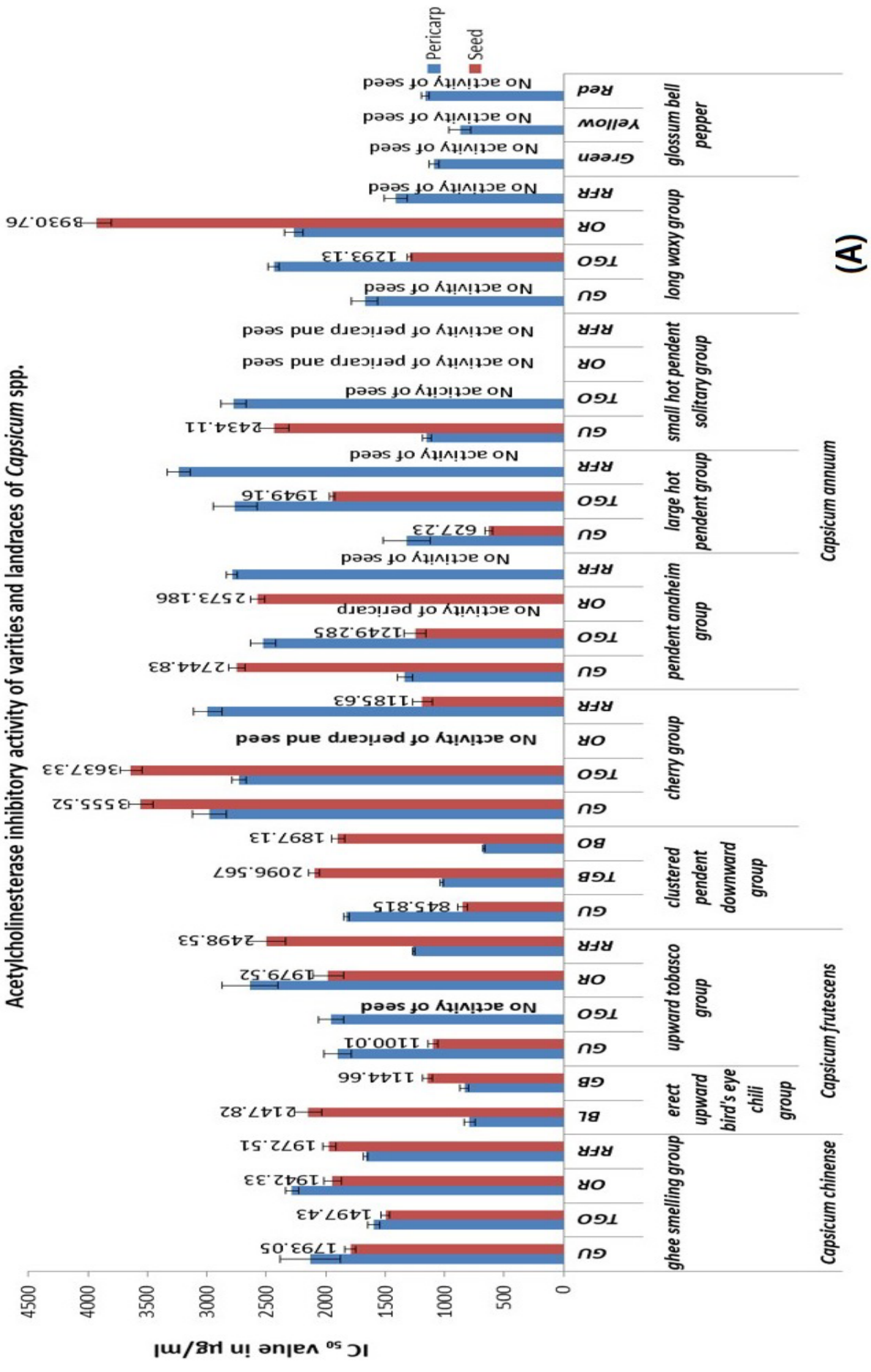
***Capsicum chinense*:** In pericarp, only the GU stage showed anti-protein denaturation activity with 98.27 ± 0.18% and all the other stages showed below 50% inhibitory activity which were considered as no activity. On the contrary, the seeds of all the four stages showed inhibition with the highest activity was shown by the seeds of OR stage with 90.0 ± 0.24% inhibition.

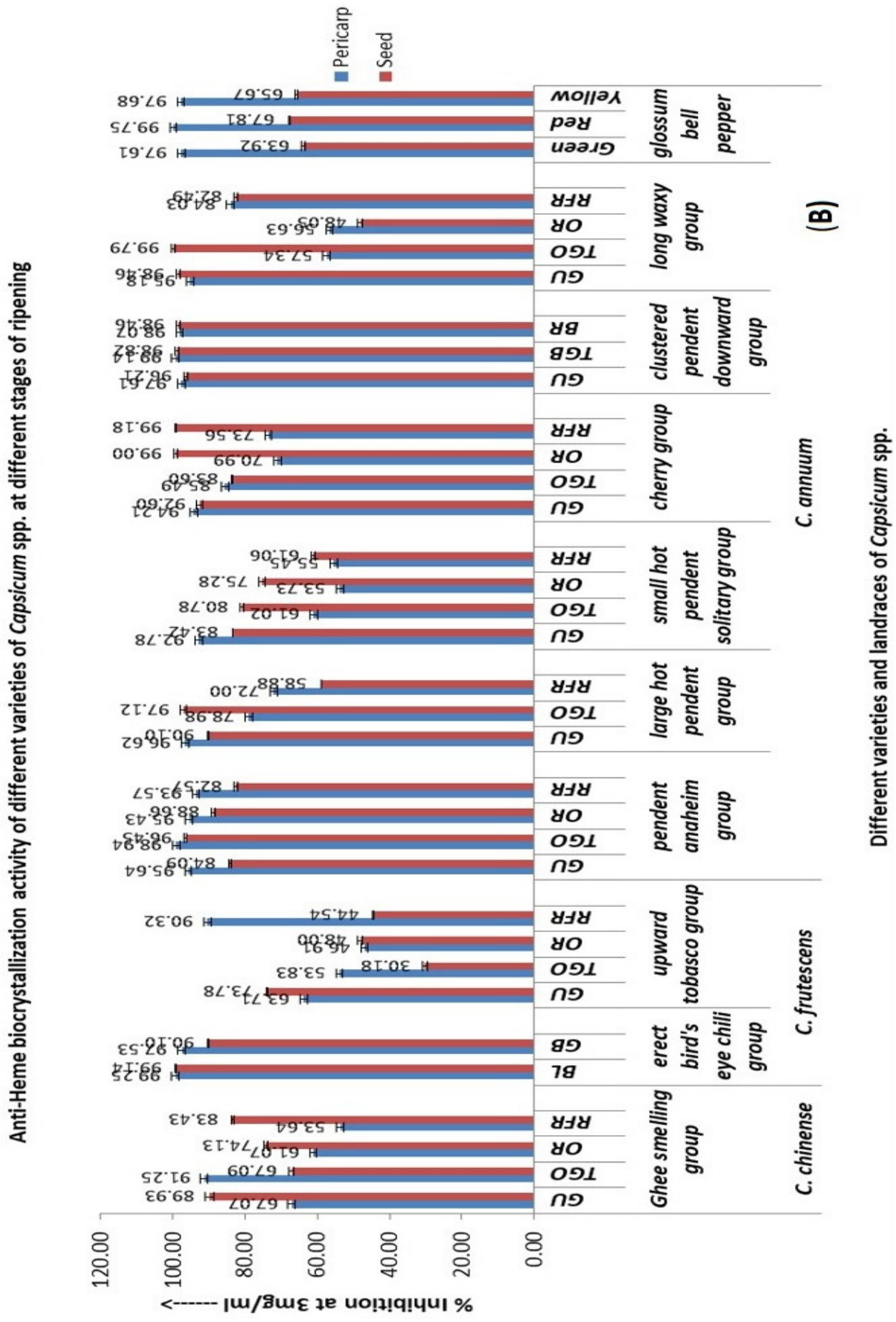
***Capsicum frutescens*:** The pericarps of BL stage of erect upward bird's eye chilli group exhibited notable anti-protein denaturation activity, *i.e.*, 99.23 ± 0.16%. The seeds of GB stage of this particular cultivar showed only 51.05 ± 0.34% inhibition. In case of upward tobacco group, only the pericarp of the OR stage showed its activity against denaturation of protein with inhibition of 77.82 ± 0.00%.

Capsicum annuum: Out of the four ripening stages, only the GU stage of the pericarp of pendent Anaheim group showed 67.84 ± 0.21% inhibition, whereas, all the stages except the OR stage of the seeds showed inhibition with the highest inhibition was measured at 88.10 ± 0.27% in the GU stage. According to our findings, no activity was detected in the large hot pendent group, neither in the pericarp nor in the seeds. Only the seeds of RFR stage of small hot pendent solitary group showed mild inhibition of protein denaturation (59.84 ± 0.25%) activity. Only the seeds of GU stage of cherry group showed inhibition with 65.49 ± 0.20%. Seeds of both GU and RFR stages of clustered pendent downward group demonstrated high activity with 91.06 ± 0.17% and 82.25 ± 0.30%, respectively. Only the RFR stage of seeds of long waxy group displayed mild inhibition towards protein denaturation activity with 54.48 ± 0.12%. The pericarps of green, red and yellow glossum bell pepper and seeds of green and yellow glossum bell pepper exhibited anti-protein denaturation property with 58.57 ± 0.23%, 82.13 ± 0.21%, 79.97 ± 0.31%, 67.05 ± 0.13% and 52.11 ± 0.21%, respectively. In this regard, the percentage inhibition of all the tested samples at the mentioned concentration is shown in Fig. 2(C).

3.4. Estimation of Capsaicin and dihydrocapsaicin content

Capsaicin and dihydrocapsaicin content were measured for each sample using HPLC-DAD. Accordingly, the highest capsaicin content (657.41 ± 112.95 µg/mg) was noted in the seeds of TGO stage followed by pericarp of GU (565.62 ± 206.69 µg/mg), TGO (524.24 ± 295.42 µg/mg) and RFR stage (357.21 ± 226.42 µg/mg) of ghee smelling group of *Capsicum chinense*. RFR and OR stage of seed as well as GU stage of pericarp of the upward tobacco group contained the second highest range of capsaicin content of 268.12 ± 10.46, 260.84 ± 6.96 and 257.51 ± 25.24 µg/mg, correspondingly.


Acetylcholinesterase inhibitory activity of varities and landraces of Capsicum spp.
IC50 value in µg/ml
Pericarp
Seed
Names of varieties and landraces of Capsicum spp.
(A)
Capsicum chinense
Capsicum frutescens
Capsicum annuum
ghee smelling group
erect upward bird's eye chili group
upward tobasco group
clustered pendent downward group
cherry group
pendent anaheim group
large hot pendent group
small hot pendent solitary group
long waxy group
glossum bell pepper
GU
TGO
OR
RFR
BL
GB
BO
TGB
Red
Yellow
Green
1793.05
1497.43
1942.33
1972.51
2147.82
1144.66
1100.01
No activity of seed
1979.52
2498.53
845.815
2096.567
1897.13
3555.52
3637.33
No activity of pericarp and seed
1185.63
2744.83
1249.285
No activity of pericarp
2573.186
No activity of seed
627.23
1949.16
2434.11
No activity of pericarp and seed
1293.13
8930.76


Anti-Heme biocrystallization activity of different varieties of Capsicum spp. at different stages of ripening
Pericarp
Seed
% Inhibition at 3mg/ml ------>
120.00
100.00
80.00
60.00
40.00
20.00
0.00
C. chinense
C. frutescens
C. annuum
(B)
Ghee smelling group
erect bird's eye chilli group
upward tobasco group
pendent anaheim group
large hot pendent group
small hot pendent solitary group
cherry group
clustered pendent downward group
long waxy group
bell glossum pepper
GU
TGO
OR
RFR
BL
GB
BR
TGB
Green
Red
Yellow
Different varieties and landraces of Capsicum spp.

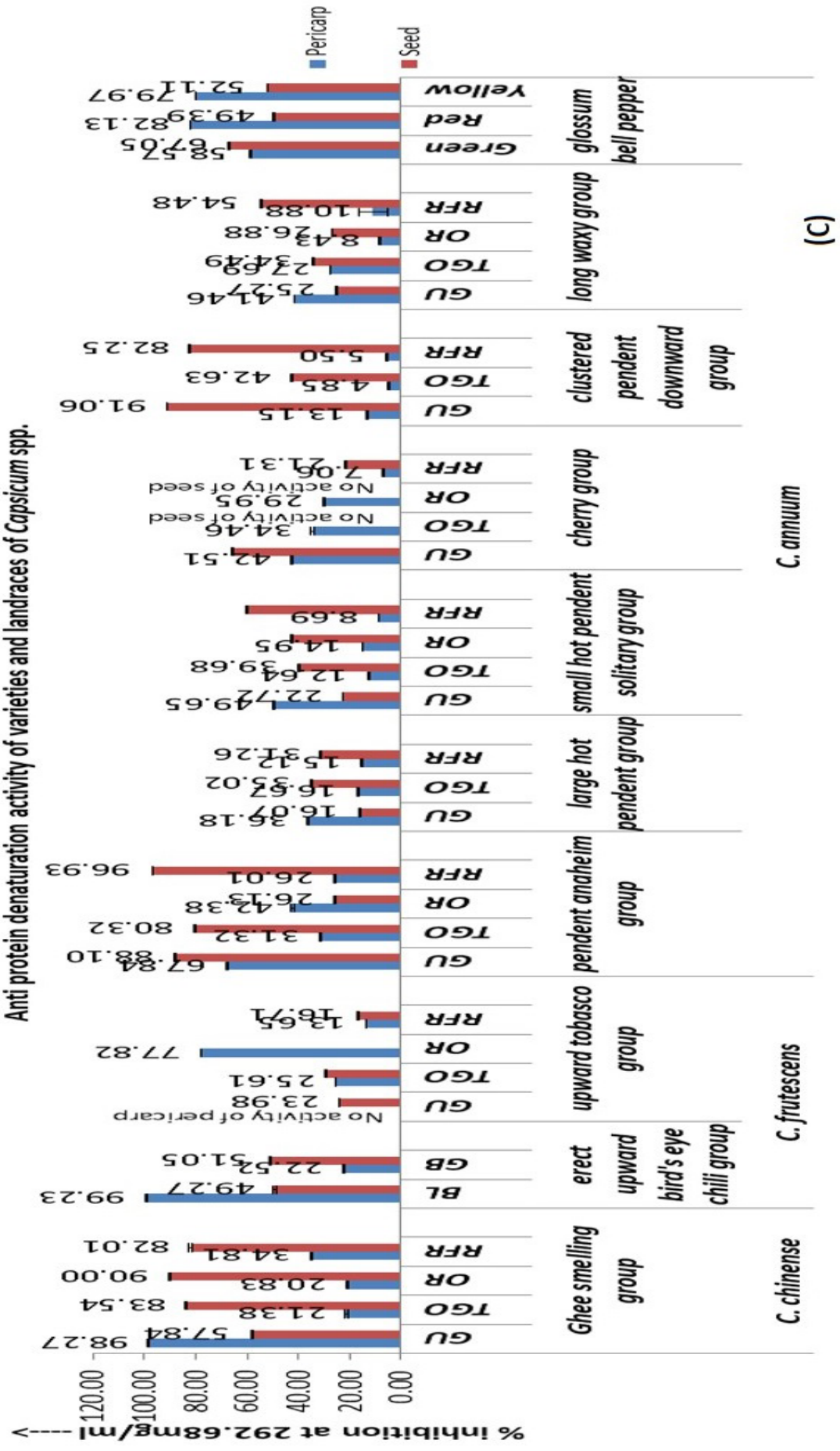


Fig. 2. (A) Acetylcholinesterase inhibitory activity; **(B)** Anti-hemebiocrystallization activity and **(C)** Anti-protein denaturation activity of different varieties and landraces of *Capsicum* sp. at different stages of ripening.

Dihydrocapsaicin was found to be excessively higher in ghee smelling group of *Capsicum chinense* than in any other groups tested. Pericarp of GU (192.27 ± 15.67 µg/mg), TGO (204.33 ± 122.60 µg/mg) and OR stage (173.92 ± 75.89 µg/mg) and seeds of TGO (121.35 ± 16.65 µg/mg) and OR stage (155.41 ± 5.65 µg/mg) of ghee smelling group contain high amount of dihydrocapsaicin. The ghee smelling group is a very hot variety of chilli that belongs to the same species as that of Bhutjolokia or "ghostpepper" whose scoville heat units (SHUs) are more than 1 million. Bosland and Baral (2007) reported that there may have been a genetic introgression from *C. frutescens* to bhutjolokia. Our study reveals that the ghee smelling variety, a probable relative of bhutjolokia of *C. chinense* has capsaicin and dihydrocapsaicin content highest among all the groups analyzed. The excessive hotness and pungency may be due to the high amount of these capsaicinoids present in the ghee smelling group. The full profile of capsaicin and dihydrocapsaicin content of all the samples at different ripening stages is presented in Table 2.

3.5. HPTLC analysis of carotenoids from *C. chinense*

The ghee smelling variety of the *C. chinense* group of chilli peppers studied here is not widely consumed by the people of West Bengal. We tried to find out the quantity of carotenoids present in the pericarp of the fruit at different stages of ripening. The amount of β-carotene, capsanthin and lutein present in each stage of ripening of ghee smelling variety of *C. chinense* reveals that the GU (β-carotene: 255.97 ± 3.52; lutein: 46.86 ± 10.97 µg/mg) and the RFR stages (β-carotene: 167.57 ± 40.13; capsanthin: 921.22 ± 311.25; lutein: 30.05 ± 13.52 µg/mg) contain good amounts of the carotenoids. The carotenoid content of all the ripening stages of the pericarp of ghee smelling variety is calculated and depicted in Table 3.

3.6. GC-MS based metabolomics and chemometric analyses

GC-MS based metabolite profiling enabled identification of a total of 118 metabolites (Supplemtary material 1 and Supplementary material 2) with the presence of several sugars and polyols, organic acids, amino acids, fatty acids, phenols and other metabolites from the pericarp and seeds of different varieties and landraces of *C. annuum, C. frutescens* and *C. chinense* at different ripening stages. PLS-DA (Partial least squares-discriminant analyses) segregated each ripening stage on the basis of their metabolite profiles. The PLS-DA 2D scores plot of both pericarp and seed (Fig. 3) show distinct separation of the ripening stages.

Among the 118 metabolites studied, some of the compounds were found to have significant correlation to anti-cholinesterase, anti-heme biocrystallization and anti-protein denaturation activities. The correlated compounds were subjected to three corresponding assays which revealed that a number of phenols have inhibitory activities. Those compounds were compared to the standard marketed drugs. The phenolic compounds identified in the experimental *Capsicum* species and landraces showing bioactivity are presented in Table 4.

Out of several methodologies, one approach to treat neurodegenerative disorders like Alzheimer's disease, insomnia, memory loss, Parkinsonism and others, is to inhibit the enzyme acetylcholinesterase which breaks down the neurotransmitter like acetycholine, needed for neurotransmission and ultimately causes impairment in cholinergic transmission. So, in this research, we inspected the compounds found in the specific ripening stages of a very common spice crop, *Capsicum*. From the Table 4, it is evident that there are several compounds which have IC_{50} values less than that of known standard drugs / inhibitors, *in vitro*. Ilkay et al. (2007) revealed that they have subjected gallic acid, chlorogenic acid and caffeic acid to AChE inhibition at a concentration of 1 mg/mL. In this study, only gallic acid showed approximately 15% inhibition to AChE while the other two compounds did not show inhibition at 1 mg/mL. Furthermore, we have successfully found out the IC_{50} values of all the three compounds, where we see that gallic acid has an IC_{50} value (32.92 ± 2.16 µM) close to the standard inhibitor galantamine hydrobromide (22.38 ± 0.44 µM). Though the other two compounds chlorogenic acid (219.18 ± 9.84 µM) and caffeic acid (687.35 ± 5.39 µM) had inhibited the AChE enzyme, their IC_{50} values are much higher than that of the standard drug.

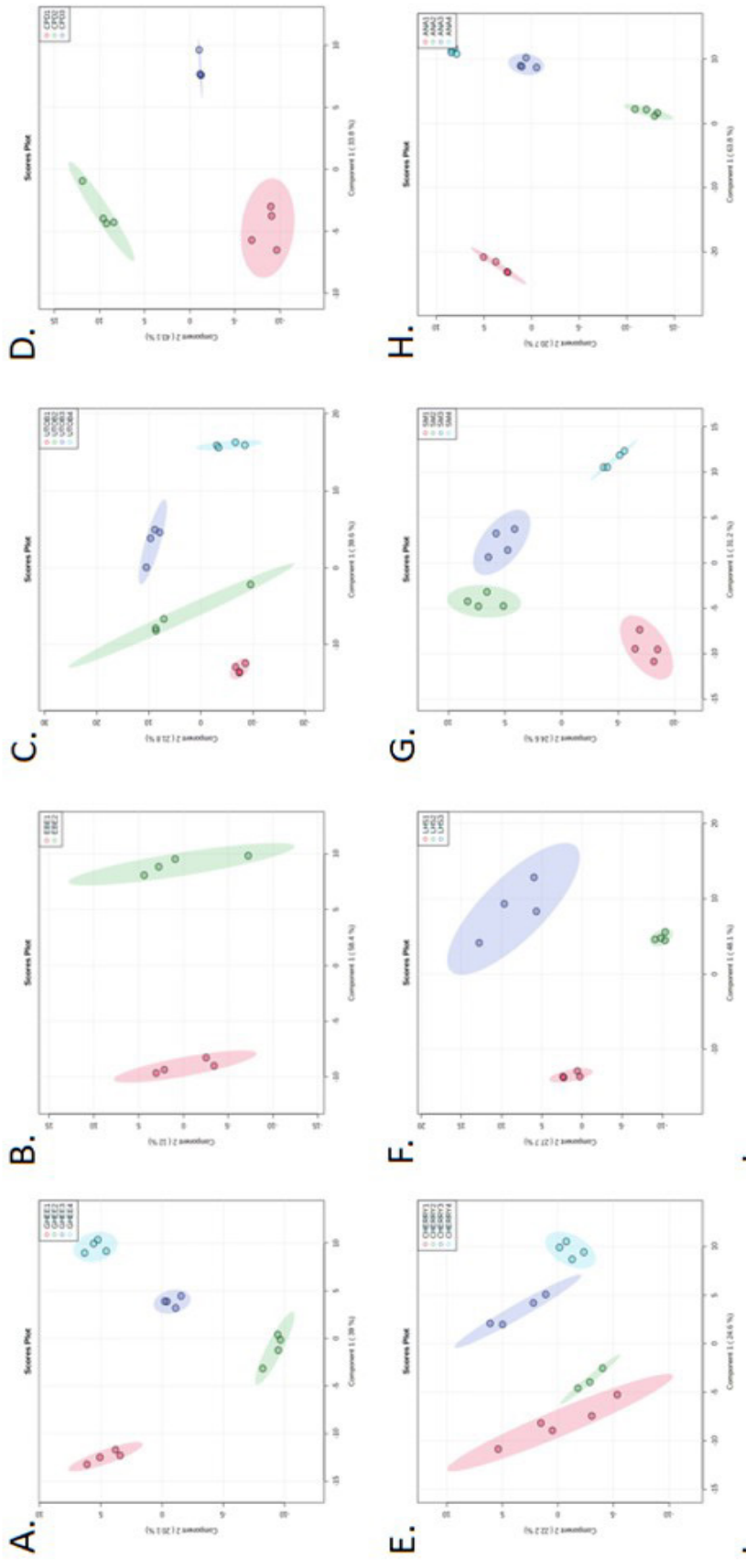
The *Plasmodium* parasite utilizes the host haemoglobin as its source of nutrition. As the haemoglobin degrades, it renders large amount of toxic heme in the host cell. The parasite converts heme monomers to insoluble cyclic dimers of hemozoin to detoxify the environment. The spectrophotometric assays to screen alternative and novel plant based anti-malarials employ hemozoin, the malarial pigment as the drug target. Our study revealed four phenolic compounds that inhibit the heme biocrystallization, namely gallic acid, chlorogenic acid, vanillic acid, and benzene 1,2,4-triol for the first time. Sharifi-Rad et al. (2022) studied the chemical composition of different *Artemisia* spp. and reported the presence of caffeic acid, chlorogenic acid, vanillic acid and different derivatives of benzene. *Artemisia* is a popular genus known for its pharmacological importance, especially anti-malarial and anti-insecticidal properties. Aldulaimi et al. (2017) tested gallic acid analogues to validate anti-malarial action of phenolic acids and revealed that the conjugates of gallic acid showed moderate activity. A group of researchers (Fordjour et al., 2020) working at the University of Ghana, reported that gallic acid was found to have highest potential for anti-malarial action, while chlorogenic acid responded moderately to *in vitro* anti-malarial drug test. Based on this study, it can be said that chlorogenic acid (IC_{50} = 0.33 ± 0.001 µM) has very close rather better anti-heme biocrystallization activity than the standard drug, chloroquine diphosphate (IC_{50} = 0.65 ± 0.045 µM) (Table 4). This study reveals two more compounds that can act as potential anti-malarial agent by inhibition of heme biocrystallization, namely vanillic acid (IC_{50} = 270.55 ± 4.82 µM) and 1,2,4-benzene triol (IC_{50} = 420.66 ± 2.02 µM) which are newly reported by our research group.

Table 2
Capsaicin and dihydrocapsaicin content of different *Capsicum* spp. fruits at different stages of ripening.

Capsicum species	Variety	Ripening stages	Capsaicin content (µg/mg) (Mean ± SD)		Dihydrocapsaicin content (µg/mg) (Mean ± SD)	
			Pericarp	Seed	Pericarp	Seed
C. chinense	Ghee smelling group	GU	565.62±206.69	199.45±136.51	192.27±15.67	72.49±18.78
		TGO	524.24±295.42	657.41±112.95	204.33±122.60	121.35±16.65
		OR	141.11±61.18	184.23±50.88	173.92±75.89	155.41±5.65
		RFR	357.21±226.42	118.69±7.64	78.86±24.39	22.76±1.32
C. frutescens	Erect upward bird's eye chilli group	GB	2.84±0.53	23.04±1.37	7.92±0.26	11.70±0.41
		BL	28.64±1.83	5.52±2.17	13.73±0.37	17.47±0.22
	Upward tobacco group	GU	257.51±25.24	191.43±16.04	53.41±7.75	33.68±1.69
		TGO	32.44±10.29	3.69±1.62	21.85±2.63	10.05±0.15
		OR	97.03±1.97	260.84±6.96	27.21±1.62	44.77±0.69
		RFR	95.92±6.89	268.12±10.46	29.11±9.28	45.32±2.06
C. annuum	Clustered pendent downward group	GU	10.05±4.26	106.08±25.05	17.02±0.80	37.84±3.98
		TGB	12.79±12.51	15.98±14.63	18.63±2.03	17.09±2.69
		RFR	9.37±5.75	38.14±4.30	21.90±1.15	23.79±0.88
	Cherry group	GU	20.42±3.19	23.34±3.02	13.43±0.53	23.03±1.19
		TGO	11.71±1.30	5.07±2.82	9.98±0.61	18.06±2.37
		OR	28.64±2.99	44.14±10.87	24.30±0.78	23.24±2.05
		RFR	5.82±0.81	22.82±0.23	10.08±0.45	41.85±1.50
	Large hot pendent group	GU	17.28±1.42	16.01±2.72	12.30±0.65	12.09±7.54
		TGO	5.12±1.56	36.24±1.22	9.57±0.84	11.04±0.21
		RFR	11.97±0.78	12.88±4.73	9.75±0.51	8.13±0.87
	Small hot pendent group	GU	27.09±2.09	23.79±7.53	16.63±0.11	26.09±2.59
		TGO	21.29±2.10	40.62±13.53	17.31±0.92	33.14±4.42
		OR	10.28±1.10	51.25±9.36	9.63±0.58	36.11±2.21
		RFR	3.58±0.86	31.29±0.99	8.55±0.64	14.33±0.71
	Anaheim group	GU	6.90±1.13	18.51±0.05	3.63±0.50	13.97±0.23
		TGO	5.82±0.13	5.32±0.88	4.10±0.18	2.91±0.73
		OR	7.61±0.04	10.87±1.38	4.29±0.05	3.75±097
		RFR	8.37±0.11	86.50±0.57	4.60±0.01	67.10±3.13
	Long waxy sweet group	GU	Not detected	Not detected	Not detected	Not detected
		TGO				
		OR				
		RFR				
	Bell peppers	Green	Not detected	Not detected	Not detected	Not detected
		Yellow				
		Red				

Table 3
Carotenoid content of Ghee smelling variety of *C. chinense*.

C. chinense	Content in µg / mg (Mean ± SD) of plant sample		
Ripening stages	β-Carotene	Capsanthin	Lutein
GU	255.97±43.52	Not detected	46.86±10.97
TGO	47.11±5.25	135.21±13.00	7.19±7.32
OR	21.30±1.90	136.35±49.82	2.10±1.64
RFR	167.57±40.13	921.22±311.25	30.05±13.52



PLS-DA 2D scores plot of pericarp of A. Ghee smelling group, B. Upward erect bird's eye group, C. Upward tobasco, D. Clustered pendent downward group, E. Cherry group, F. Large hot pendent group, G. Small hot pendent group, H. Anaheim group, I. Long waxy group, J. Glossum bell pepper.

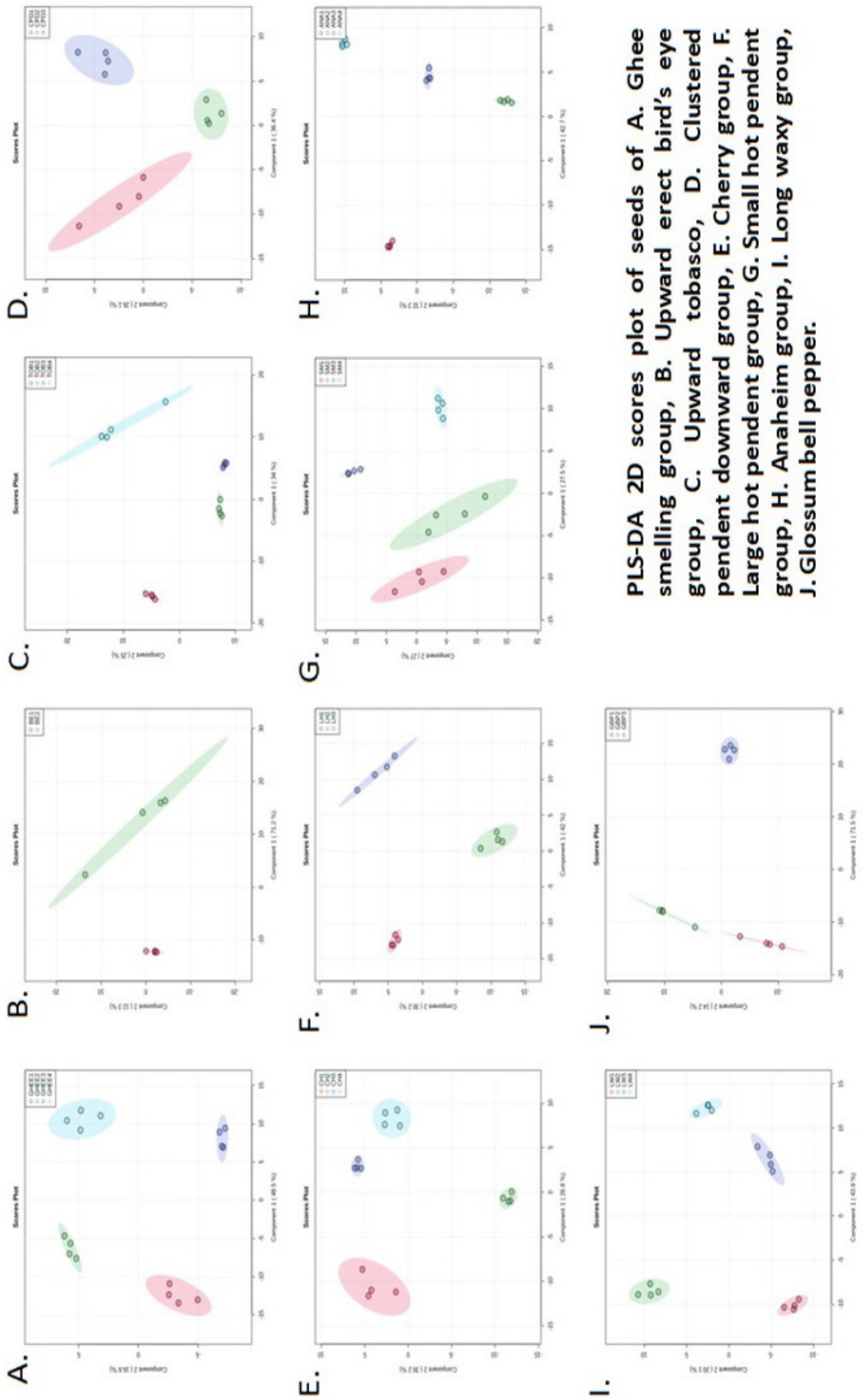


Fig. 3. PLS-DA 2D scores plot of pericarps and seeds of different varieties of *Capsicum* sp. showing segregation of different ripening stages.

Table 4
IC_{50} values of correlated compounds and comparison with standard drugs.

	Names of compounds	IC_{50} (µM±SD)
Compounds correlated to AChE inhibitory assay	Galantamine hydrobromide (Standard drug)	22.38±0.44
	Gallic acid	32.92±2.16
	1,2,4-Benzene triol	32.79±0.93
	Chlorogenic acid	219.18±9.84
	Caffeic acid	687.35±5.39
Compounds correlated to anti-heme biocrystallization assay	Chloroquine di phosphate (Standard Drug)	0.65±0.045
	Chlorogenic acid	0.33±0.001
	Vanillic acid	270.55±4.82
	Benzene1,2,4-triol	420.66±2.02
	Gallic acid	565.35±16.18
Compounds correlated to anti-protein denaturation assay	Ibuprofen (Standard Drug)	96.95±0.17
	O-Acetylsalicylic acid	4.50±0.15
	4-Hydroxybenzoic acid	7.63±0.06
	Quinic acid	5.56±0.19
	Capsaicin	7.25±0.08
	3,4-Dihydroxybenzoic acid	14.83±0.31
	Vanillic acid	15.64±0.26

The denaturation of proteins as one of the causes of inflammation is well-documented. Production of auto-antigens in certain rheumatic diseases may be due to *in vivo* denaturation of proteins. A number of anti-inflammatory drugs are known to inhibit the denaturation of proteins. Ali et al. (2012) concluded that presence of phenolic compounds increases the thermal stability of proteins because phenolic interactions change secondary structure of proteins. In the present study, a number of phenolic compounds identified from the metabolic profile that had correlation with anti-protein denaturation activity, were tested for the same assay individually. *O*-acetyl salicylic acid (IC_{50} = 4.50 ± 0.15 mM), 4-hydroxy benzoic acid (IC_{50} = 7.63 ± 0.06 mM), 3,4-dihydroxy benzoic acid (protocatechuic acid) (IC_{50} = 14.83 ± 0.31 mM), quinic acid (IC_{50} = 5.56 ± 0.19 mM) and vanillic acid (IC_{50} = 15.64 ± 0.26 mM) significantly inhibited protein denaturation *in vitro*. Capsaicin is a US-FDA approved neuropeptide-active agent used in the treatment of pain related to arthritis and musculoskeletal pain. It has the capacity of depleting supply of substance P to the nerves. Substance P is a neuropeptide that acts as a chemical mediator from peripheral to the central nervous system. Depletion of this neuropeptide prevents transmission of impulses to the brain and renders joints insensitive to the pain feeling. The function of substance P is involved in the pathogenesis of various diseases including but not limited to cancer, diabetes, rheumatoid arthritis, myocarditis, heart failure, epilepsy, migraine, thrombosis, pruritus, depression, and anxiety (Graefe and Mohiuddin, 2022). Capsaicin is the pungent ingredient of chilli peppers and is approved as a topical treatment of neuropathic pain. The analgesia lasts for several months after a single treatment. Capsaicin selectively activates TRPV1, a Ca^{2+}-permeable cationic ion channel that is enriched in the terminals of certain nociceptors (Chung and Campbell, 2016). Therefore, capsaicin is also tested for the same anti-protein denaturation assay and the *in vitro* 50% inhibitory concentration was obtained which was significantly lower (IC_{50} = 7.25 mM ± 0.08) than the standard drug Ibuprofen (IC_{50} = 96.95 ± 0.17 mM) tested.

4. Concluding remarks

This study has provided valuable insights into the anti-cholinesterase, anti-heam biocrystallization and anti-arthritic potentialities of both non-pungent sweet (long waxy group and green, yellow and red glossum bell pepper) as well as pungent hot *Capsicum* [*Capsicum chinense* (ghee smelling group), *C. frutescence* (erect upward bird's eye chilli group, upward tobacco group) and *Capsicum annuum* (clustered pendent downward group, cherry group, pendent Anaheim group, large hot pendent group, small hot pendent solitary group)]

fruits with special emphasis on their ripening stages. Since they are consumed all over the world at different ripening stages, they can be generally considered safe. The selection of proper ripening stages may serve as potential leads to drug targets for neurodegenerative diseases, malaria and arthritis. The phenolic compounds which were correlated and further assayed in this work may be substantiated through *in vivo* models.

Cultivation and consumption of *Capsicum* fruits should be encouraged more. Apart from the biological activities shown by the plant samples, this study provides supplementary information on chemotaxonomic significance and baseline data for the preference of suitable genotypes in plant breeding based on nutritional and pharmacological importance. The study may be beneficial in selecting the cultivars with the best attributes.

Author contribution statement

Conceptualization and literature search were performed by Mamita Debnath and Jhelam Chatterjee. The first draft of the manuscript was prepared by Susmita Das. Susmita Das critically analyzed and gave suggestions to finalize the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they donot have any conflicts of interest.

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Original Research Article

Simultaneous optimization of extraction of bioactive compounds and antioxidant activity of *Ammi visnaga* (L.) Lam aerial parts using response surface methodology

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ABSTRACT

In this report, different extracts from the aerial parts of *Ammi visnaga* (L.), *e.g.*, flowers, leaves, and stems were prepared using water, methanol, and ethanol. To optimize the extraction process, the design of mixtures was carried out using different extracting solvents and their combinations. The special cubic model explained the variance of the TPC and the antioxidant activity of the extracts at a level of R^2 > 95%. In general, the analysis of the model-derived response surfaces revealed that in binary mixtures (50% ethanol + 50% methanol), the yielded values of phenolic compounds and the antioxidant activity increase with the water proportion of different prepared mixtures. The ability of the quaternary mixture to extract the phenolic compounds was also positively and significantly influenced by the water content, creating a mild polar medium for the extraction of phenolic compounds. The phenolic profile of different extracts under study revealed the presence of a cocktail of active ingredients, including chlorogenic acid, caffeic acid, rutin, *p*-coumaric acid, etc. especially the flower extract of *A. visnaga* (L.).

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1. Introduction

The Umbelliferae family, also known as Apiaceae, encompasses a diverse group of plants with approximately 434 genera and nearly 3,780 species distributed across various habitats worldwide. These plants are economically significant, serving as leaf and root vegetables, herbs, spices, and ornamentals (Spinozzi et al., 2021). The family is characterized by aromatic herbs with distinctive feather-divided leaves and flowers arranged in umbels. While many species are utilized for culinary purposes, some members like poison hemlock and water hemlock are poisonous. Additionally, plants such as carrot, celery, parsley, and fennel are commonly used as vegetables, while others like anise, coriander, and cumin are valued for their herbal and spice properties (Teng et al., 2023). The Umbelliferae family's rich diversity and economic importance underscore its relevance in both traditional and modern applications (Spinozzi et al., 2021; Teng et al., 2023; Valatabar et al. 2023). *Ammi visnaga* (L.) Lam. belongs to the Umbellifereae family distributed natively in the Nil Valley, North Africa, Europe, Asia, and North America (Franchi et al., 1985; El Jabboury aet al. 2023). For centuries, humans have developed their knowledge in pharmacognosy and phytochemistry, which is considered a cornerstone of traditional and modern medicine (Mohammadhosseini et al. 2019a; Mohammadhosseini et al. 2019b; Awuchi, 2023; Chaniad et al., 2023; Theodoridis et al., 2023). Medicinal herbs are widely cultivated for many purposes including extraction of bioactive compounds with biological activities, *e.g.*, antidiabetes, antiobesity, antiinflammation, and antimicrobial effects (Nwozo et al., 2023). These plants have been used in folk medicine to treat renal colic, abdominal cramps, vitiligo, and psoriasis (Khalil et al., 2020). Medicinal plants are also considered an inexhaustible source of

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phenolic compounds well-known for their biological properties (Molin et al., 2014). The beneficial properties of *A. visnaga* (L.) are attributed to its dense chemical composition. The phytochemical compounds of *A. visnaga* (L.) include pyrones, coumarins, khellin, visnagin, 4-norvisnagin, khellinol, visamminol, ammiol, and khellol (Abou-Mustafa et al., 1990; Hashim et al., 2014). The aerial parts of this plant are rich in phenolic compounds in aglycone and conjugated forms (Khalil et al., 2020). The delve into the phytochemistry of the plant (*A. visnaga* (L.)) revealed that quinic acid was the most abundant bioactive compound (9.436 mg/g) followed by other compounds, including gallic acid, protocatechuic acid, and gentisic acid (El-guourrami et al., 2023). FTIR analysis indicats that *A. visnaga* (L.) contains long-chain linear aliphatic compounds, lipids, amides, and aromatic components (Benabderrahmane et al., 2023). Robust evidence found that the lower doses of *A. visnaga* (L.) extracts did not cause any sign of toxicity and the LD_{50} for intraperitoneal and oral administration of the herb extract was found to be 3.6 and 10.1 g/kg, respectively (Jouad et al., 2002; Koriem et al., 2019).

The health benefits of *A. visnaga* (L.) have been investigated by several studies highlighting its antidiabetic, antihyperlipidemic, anticancer, antibacterial, and antifungal activities (Koriem et al., 2019). Bioactive compounds are found in different amounts in natural products and used extensively in pharmaceutical and food industries. Thus, the main challenge is likely to find the most appropriate method(s) to obtain the highest yield of these compounds well-known for their pharmacological properties (Lin et al., 2018). Different techniques were used to evaluate their ability to extract the maximum content of phenolic compounds comprising mild methods like ultrasound-assisted extraction (UAE), microwave assisted extraction (MAE), pressurized liquid extraction (PLE), pulsed electric field (PRF) pretreatment, ohmic heating (OH) pretreatment, and cold plasma (CP) pretreatment as well as conventional methods, *e.g.*, solvent extraction, soxhlet extraction, squeezing or cold pressing, and steam distillation (Ebrahimi and Lante, 2022). Recently, several investigations have been carried out to examine efficient extraction techniques to reduce extraction time, energy and costs, along with organic solvent consumption (Lopez-Avila and Luque de Castro, 2014). It has been proved that flavonoids and terpenes are both successively extracted using ethyl alcohol and ethyl acetate, while polyphenols can be extracted by a mixture of acetone, methanol, and ethanol (Alberti et al., 2014). The extraction optimization using different extracting solvent mixtures could provide promising findings (Pourbasheer et al. 2014; 2017). The design of experiments has highlighted its importance for the extraction optimization process (Cavalcanti et al., 2021). Response surface methodology (RSM) is a valuable tool extensively utilized to optimize the extraction of phenolic compounds. It aids in predicting the most suitable combination of extracting solvents, thereby minimizing time, solvent usage, and reducing the need for extensive benchwork (Cavalcanti et al., 2021).

In the present report, response surface methodology was used to optimize the extraction of phenolic compounds and antioxidant activity using three complementary assays, namely HCA, TAC, and CUPRAC as well as the phenolic profile from the aerial parts of Moroccan A. *visnaga* (L.) collected from Taounate region.

2. Experimental

2.1. Plant material

The wild fresh plant (Fig. 1) was collected in the Taounate region of Morocco (34°33'47'N, 4°39'34'W) in April 2020, according to the guidelines described by the IUCN Policy Statement on Research Involving Species at Risk of Extinction and the Convention on the Trade in Endangered Species of Wild Fauna and him Flora (IUCN, 1989). The plant parts, viz., flowers, leaves, and stems were separated and subsequently dried at 40 ºC and finely ground before extraction process. The plant was identified by the Team of the Department of Botany and Vegetal Ecology of the Scientific Institute, Rabat. A representative sample of the plant material (*A. visnaga* (L.)) was deposited at the herbarium of the same department under voucher number RAB114158.

Fig. 1. The photograph of *Ammi visnaga* (L.).

2.2. Extraction procedure and sample preparation

The extraction was done in triplicates using three pure solvents, water, ethanol, and methanol, and their mixtures, according to the following procedure. In this context, 50 mg of dried and pulverized inflorescence of *A. visnaga* (L.) were extracted for 20 min by sonication with 1 mL of solvents mixture. The extracts were centrifuged for 15 minutes at 6000 rpm, and the supernatants were recuperated and stored at 4 ºC (El Jabboury et al., 2022).

2.3. Evaluation of solvent impacts by simplex axial design

Two dissimilar categories of standard designs are usually used for the extraction experimentation with combinations involving (i) Simplex-centroid design, and (ii) Simplex-lattice design. Both designs assess the triangular reply surface at the vertices (*i.e.*, the corners of the triangle), then the centroids (sides of the triangle) (Montgomery, 2013). In the simplex-centroid design, different conditions tested a triangle with pure components in the vertex, representing 100% of each single solvent. Central points on every side express permutations of the binary blends (1/2: 1/2: 0; 1/2: 0: 1/2;

0: 1/2: 1/2), and, the medium point as a ternary mixture (1: 1: 1). This scheme is from time to time increased with internal points (axial ones) expressing 2/3 of one of the targeted solvents and 1/6 for the others (Fig. 2), also known as simplex axial design (SAD) (Sampaio et al., 2015). To boost the extraction procedure, a mixture design was developed as presented in Fig. 1. The simplex-centroid design coupled with axial points in three replicates was chosen to determine the solvent combination of water (W), ethanol (E) and methanol (M). Fig. 2 presents all tested conditions. This design, permitted the evaluation of linear (W, E and M), quadratic (WE, WM, and EM), and special cubic (WEM) models for the response under study.

2.4. Total phenolic content (TPC)

The total phenolic content (TPC) was determined using the Folin-Ciocalteu method described by Hasperué et al. (2016). In brief, 50 µL of the extract was mixed with 450 µL of Folin-Ciocalteu reagent (0.2 N) for 5 min, and then 450 µL of a Na_2CO_3 solution (75 g L^{-1}) was added. All samples were incubated at room temperature in dark condition for 2 h and their absorbance was read at 760 nm in a Jenway 6505 UV/visible, scanning spectrophotometer. The calibration curve was obtained using gallic acid over the concentration range of 0-250 µg/mL. The experiment was tested in triplicates and the results were expressed as mg GAE/g of dried plant.

2.5. Total dihydroxycinnamic acid derivative content (HCA)

The total HCA content was estimated using the method described by Fraisse et al. (2011). Total HCA content in the extract was determined from the calibration curve with chlorogenic acid (CGA) as standard. Results were expressed as milligrams of CGA equivalents (CGAE) per g of dried weight.

2.6. Determination of antioxidant activity

2.6.1. Total antioxidant capacity (TAC)

This assay was performed according to the procedure described by Yilar et al. (2020). Accordingly, a calibration curve was prepared using different concentrations of ascorbic acid as standard and the relevant results were expressed as mg of ascorbic acid equivalents (AAE) per g of DW.
In each method, all samples were analysed in triplicates (n = 3). Absorbance of the resulting solution was measured using a UV/visible spectrophotometer.

2.6.2. CUPRAC assay

Further elution using hexane-CH_2Cl_2 (95:5, *v/v*) afford a white powder of (**4**)**,** yield 10.6 mg; m.p. 302-304 ºC; ^{13}C-NMR (CD_2Cl_2; 125MHz): δ 154.8 (C-20), 107.1 (C-30), 80.9 (C-3), 55.4 (C-5), 51.0 (C-9), 48.8 (C-18), 41.9 (C-14), 41.2 (C-8), 39.6 (C-19), 38.8 (C-16), 38.7 (C-13), 38.2 (C-22), 38.1 (C-1), 37.7 (C-4), 37.0 (C-10), 34.7 (C-17), 33.9 (C-7), 27.9 (C-23), 26.6 (C-15), 26.1 (C-12), 25.6 (C-29), 25.5 (C-21), 23.6 (C-2), 21.4 (C-11), 19.8 (C-28), 18.1 (C-6), 16.4 (C-24), 16.3 (C-26), 15.8 (C-25), 14.7 (C-27) (Trinh et al., 2008).

2.7. Phenolic profile

The dionex Ultimate 3000 UHPLC system (ThermoFisher Scientific, Bremen, Germany) equipped with a diode array detector (DAD) and TSQ Quantum Access Max triple-quadrupole (QQQ) mass spectrometer (ThermoFisher Scientific, Basel, Switzerland) was used to delve into the phytochemistry of different parts of *A. visnaga* (L.). Elution was performed at 40 °C on a Syncronis™ C18 column (100 × 2.1 mm) with 1.7 µm particle size (ThermoFisher Scientific, Fair Lawn, NJ, USA). Water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) were used as the mobile phase following gradient elution: 5% B in first min, 1-14 min 5-95% B, 14-14.2 min 95-5% B and 5% B until the 20th min (Skorić et al., 2022). Full scanning (FS), product ion scanning (PIS) and neutral loss scanning (NLS) modes were conducted for qualitative analysis. Xcalibur software (version 2.2) was used for the instrument control, data acquisition, and analysis. Compounds were identified by direct comparison with commercial standards and literature data. The total amount of each compound was evaluated by calculation of peak areas (Skorić et al., 2022).

2.8. Statistical analysis

The models described above, utilizing a 3rd-degree polynomial function, were used in surface matching. The effect of S-LD on TPC and antioxidant activity was analyzed using the least square multiple regression method. According to this experiment, typical multiple regression equations were used as follows:

Linear model: $y = b_1 \times x_1 + b_2 \times x_2 + b_3 \times x_3$ (Eqn. 1)

Quadratic model: $y = b_1 \times x_1 + b_2 \times x_2 + b_3 \times x_3 + b_{12} \times x_1 \times x_2 + b_{13} \times x_1 \times x_3 + b_{23} \times x_2 \times x_3$ (Eqn. 2)

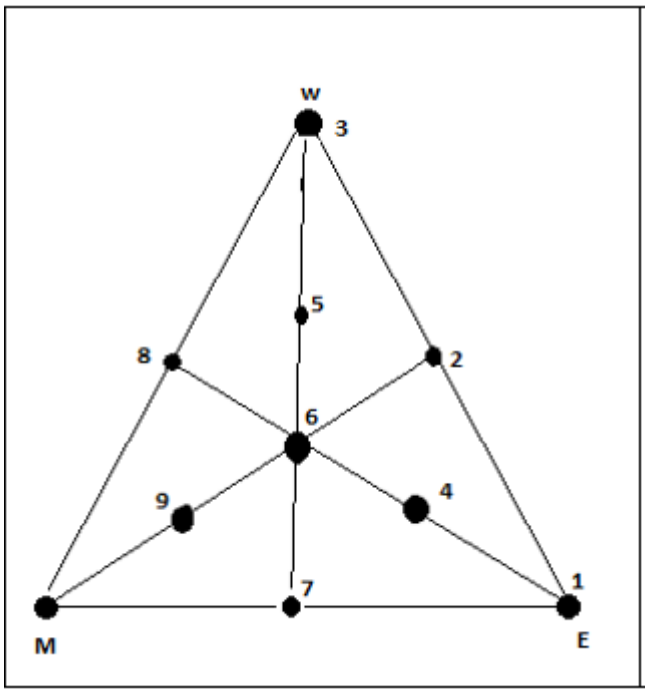
Special cubic model: $y = b_1 \times x_1 + b_2 \times x_2 + b_3 \times x_3 + b_{12} \times x_1 \times x_2 + b_{13} \times x_1 \times x_3 + b_{23} \times x_2 \times x_3 + b_{123} \times x_1 \times x_2 \times x_3$ (Eqn. 3)

The following parameters: Sum of square (SS), mean of square (MS), df (degree of freedom), test F, *p*-values, R-square (R^2), R-square adjusted (R^2_{adj}). were considered when selecting the appropriate statistical model.
The analysis of variance (ANOVA) was applied to determine the fitness of the multiple regression model ($p < 0.05$) and to evaluate the significant effects of variables and the relevant interactions. The analyses were performed using the free version of STATISTICA version 10 software.

3. Results and Discussion

3.1. Total phenolic content

The results of total phenolic compounds obtained for extracts of the four parts of *A. visnaga* (L.) are given in Table 1.
The process of recovering the phenolic compounds in multiple samples is persuaded by the solubility of these compounds and the polarity of the solvent applied in



	Ethanol	Water	Methanol
1	0.00	0.00	100.00
2	0.00	50.00	50.00
3	0.00	100.00	0.00
4	16.67	16.67	66.67
5	16.67	66.67	16.67
6	33.33	33.33	33.33
7	50.00	0.00	50.00
8	50.00	50.00	0.00
9	66.67	16.67	16.67
10	100.00	0.00	0.00

Fig. 2. Simplex axial design (SAD) (W: Water, M: Methanol, E: Ethanol).

Table 1
Total phenolic contents obtained for extracts the four parts of *Ammi visnaga* (L.).

Number	Ethanol	Methanol	Water	TPC mg GAE/g		
				Flower	Stem	Leaf
1	0.00	50.00	50.00	47.47±0.97	25.49±0.17*	27.63±0.07
2	0.00	100.00	0.00	39.25±1.19^{a}	28.61±0.06*	31.22±1.06
3	16.67	16.67	66.67	25.57±1.08^{ab}	14.55±0.14^{ab*}	17.91±0.38^{ab}
4	16.67	66.67	16.67	41.44±1.89^{c}	28.24±0.34^{c*}	28.39±0.84^{c}
5	33.33	33.33	33.33	34.21±0.16^{ac}	19.96±0.99^{d*}	24.31±0.48
6	50.00	0.00	50.00	38.75±0.36^{ac}	25.51±0.11^{c*}	31.29±0.59^{ce}
7	50.00	50.00	0.00	49.71±0.12^{bcde}	32.09±0.44^{ce*}	24.41±0.56**
8	66.67	16.67	16.67	19.01±1.1^{abde}	13.67±0.06^{abcd*}	11.81±0.38^{abde*}
9	100.00	0.00	0.00	29.73±1.91^{abd}	22.24±1.59*	20.29±2.50^{bde}
10	0.00	0.00	100	10.90±1.10 ^{abcde}	8.34±0.39^{abde}	5.84±0.27^{abcde}
All Runs				33.60±11.99^{a}	21.87±7.38*	22.31±8.17^{be}

the extraction process (Sulaiman et al., 2011). The results obtained showed that the total phenolic compound (TPC) ranged from 8.34 ± 0.39 to 32.09 ± 0.44 mg GAE/g for dry stem. However, these amounts varied greatly among flower extracts between 10.90±1.10, and 49.71 ± 0.12 mg GAE/g, and from 5.84 ± 0.27 to 31.29 ± 0.59 mg GAE/g in the leaves. This highlights the influence of the extracting solvent. A previous report has shown that the TPC amount found in *A. visnaga* (L.) ranges from 76.10 to 195.30 mg GAE/g (Aourabi et al., 2021). The obtained results are lower than those reported previously (Sarra et al., 2018; Aourabi et al., 2021). This variability is highly related to environmental factors such as ecolimatic conditions, drought, salinity, viruses among others (de Carvalho et al., 2018; Vaughan et al., 2018).

3.2. Analysis of variance (ANOVA)

Table 2, also at the model shows the significant effect of the processing variables on the TPC (p < 0.001). In

general, the effect of all processing variables was determined to be positive, which indicated that the increase in each solvent level provided an increase in total phenolic content. Optimization of TPC extraction by simplex lattice mixture design from *Prunus mahaleb* L. showed the same effect of solvent likewise our finding (Ozturk et al. 2014).

The linear model explained the variance at the level of R^2 with a frequency of (R^2 0.65 and 0.62 R^2adj), (R^2 0.65 and 0.62 R^2adj), (R^2 0.57; 0.54 R^2adj) and (R^2 0.25; 0.20 R^2adj), for leaves, flowers, stem, and roots, respectively. Expanding from linear to quadratic model improved the fitness for regression analysis (Table 3).

Table 2
Analysis of variance results for different statistical models.

Model	SS Effect	df Effect	MS Effect	F	p	R^2	R^2_{adj}
leaves							
Linear	1263.19	2.00	631.59	25.41	0.00	0.65	0.62
Quadratic	522.09	3.00	174.03	28.05	0.00	0.92	0.90
Special Cubic	77.62	1.00	77.62	25.06	0.00	0.96	0.954
Total Adjusted	1934.15	29.00	66.69				
Flowers							
Linear	3045.93	2.00	1522.96	36.67	0.00	0.73	0.71
Quadratic	952.49	3.00	317.49	45.18	0.00	0.96	0.95
Special Cubic	0.41	1.00	0.41	0.057	0.008	0.96	0.94
Total Adjusted	4167.06	29.00	143.69				
Stems							
Linear	909.58	2.00	454.79	18.27	0.00	0.575	0.54
Quadratic	624.02	3.00	208.0	104.41	0.00	0.970	0.96
Special Cubic	11.25	1.00	11.250	7.077	0.00	0.977	0.97
Total adjusted	1581.42	29.00	54.532				

Table 3
Coefficients of the overall fitness for the regression model ($p < 0.05$).

	SS	df	MS	F	*p*
Leaves					
Model	1862.92	6.00	310.49	100.24	0.00
Total Error	71.24	23.00	3.10		
Lack of Fit	52.57	3.00	17.52	18.77	0.01
Pure error	18.67	20.00	0.93		
Total adjusted	1934.16	29.00	66.70		
Flowers					
Model	3998.84	6.00	666.47	91.12	0.00
Total Error	168.22	23.00	7.31		
Lack of Fit	141.27	3.00	47.09	34.95	0.02
Pure error	26.95	20.00	1.35		
Total adjusted	4167.06	29.00	143.69		
Stems					
Model	1544.86	6.00	257.48	161.96	0.00
Total Error	36.56	23.00	1.59		
Lack of Fit	28.51	3.00	9.50	23.62	0.00
Pure error	8.05	20.00	0.40		
Total adjusted	1581.42	29.00	54.53		

Whereas, the best fitness was found in the special cubic model in which the coefficients of determination were also improved. This special cubic model also explained (R^2 0.96 and 0.95 R^2adj), (R^2 0.96 and 0.95 R^2adj), (R^2 0.98; 0.97 R^2adj) and (R^2 0.91; 0.89 R^2adj), of the variance and adjusted it for leaves, flowers, stem, and roots, respectively. similar results have been described by Baj et al. (2018). This evaluation may indicate a statistically significant interaction between three-component systems which could be explained by the higher complexity of the model and the interactions that may occur between the three selected solvents.

Due to a higher coefficient of determination, a special cubic model was chosen to further evaluate the statistical impact of the composition of the solvent mixture installed on the total phenolic content of extracts from different parts of the plant. The regression models for the experiment are shown below:

TPC-Leaves = +6.41×x + 17.86×y + 26.66×z + 0.809×x×y + 29.907×x×z + 31.79×y×z + 163.18×x×y×z+0 (Eqn. 4)

TPC-Flower = +11.19×x + 26.70×y + 45.80×z + 5.958×x×y + 79.32×x×z + 9.85×y×z - 11.92×x×y×z + 0 (Eqn. 5)

TPC-Stem = +9.03×x + 14.38×y + 24.91×z + 9.92×x×y + 60.90×x×z + 32.84×y×z - 62.12×x×y×z + 0 (Eqn. 6)

In the above equations, the symbols X, Y and Z respectively stand for ethanol, methanol and water. Furthermore, in all four parts of the plant, TPC was positively and linearly influenced by methanol (y) and water (z), respectively. The obtained results show that ethanol (x) has the lowest coefficient; and the smallest proportion of TPC.

In the group of binary interactions, the use of ethanol reduced the extraction ability of methanol (xy), without affecting the extracting power of water (z). The ternary interaction (xyz) showed a synergistic effect between the components of the mixture for leaves and roots, while it displayed an antagonistic effect on TPC extraction from flowers and plant stems.

3.3. Surface analysis

3.3.1. TPC from leaves

Total phenolic compound was determined to evaluate the strength of the extracting solvent used on different parts of *A. visnaga* (L.). The special cubic model gave a satisfactory value to determine the coefficient (R^2 = 0.963, R^2adj = 0.954), and all coefficients were significant at the 95% confidence level.

The response surfaces for TPC, which were obtained from leaves by mixture design for the percentage composition of methanol, water, and ethanol, are illustrated in Fig. 3A and Fig. 3B. As seen, water was the best solvent for the TPC extraction followed by methanol, whereas ethanol extracted the lowest amount of TPC. According to Fig. 3 (A and B), in binary mixtures, adding water to ethanol and methanol increases its power to extract TPC. The highest amount was extracted by ternary mixtures.

The best solvent mixture given by the analyzed program for optimal TPC extraction from the leaves of *A. visnaga* (L.) consisted of 50% of water, 40% methanol, and 10% of ethanol.

3.3.2. TPC from flowers

The quadratic and special cubic models explained better the variance in TPC content at the level of R^2 (0.96), indicating the well fitness of the proposed model. Thus, it is capable of proper predicting of the behavior of the mixture.

The response surfaces for TPC, which were obtained from flowers by mixture design as a function of the percentage composition of water, methanol, and ethanol, are illustrated in Fig. 4 (A and B).

In the linear interactions, water served as the best extracting solvent followed by methanol and ethanol. Moreover, the analysis of the binary interaction shows that TPC increases with the increase in water in the two solvents. The best binary interaction occurs between water and ethanol. The ternary interaction did not have a good impact on TPC extraction. The highest extract amounts were found to occur with aqueous and ethanol between 50 and 90%. It could be also inferred that water addition to ethanol and methanol could increase their power to extract TPC from flowers.

3.3.3. TPC from stems

The surface fitting for special cubic model is shown in Fig. 5 (A and B). Accordingly, water has better linear effect on TPC extraction compared to methanol and ethanol. The increasing water ratio in the binary mixture with methanol and ethanol, increases the polarity of the solvent mixtures and enhances its ability to extract phenolic compounds. The best results are seen when the water content in the mixture reaches 50%. That amount returns to decrease when water amount overtakes 75%.

3.4. Pareto chart analysis

Pareto charts show each effect and the combination of effects by a bar in decreasing order of significance. From a graphical viewpoint, this helps us visualize the influencing variables and their degree of influence. The Pareto diagrams present a ranking of the most significant factors to the significant ones, with a significance limit of the *p*-value of 5.0%. A factor with a negative effect has a higher value for the low level than the value for the high level (Chemistry and Duret 2012). Fig. 6 (Fig. 6-1, Fig. 6-2, and Fig. 6-3) shows the Pareto chart of the effects of the studied variables on the polyphenol content. Water was the solvent that influenced mostly and positively phenolic extraction from different parts of plants, followed by methanol. Ethanol was the third parameter that showed a significant positive effect for the extraction from *A. visnaga* (L.) leaves, while the binary integration between water and methanol (AC) comes in the third place, also with a positive effect for leaves, stem, and roots.

Table 4 displays the obtained results of the antioxidant activity. The analysis of the obtained experimental amounts was in high concordance with the predicted amounts, suggesting that this statistical tool revealed its efficacy to choose the most appropriate solvent

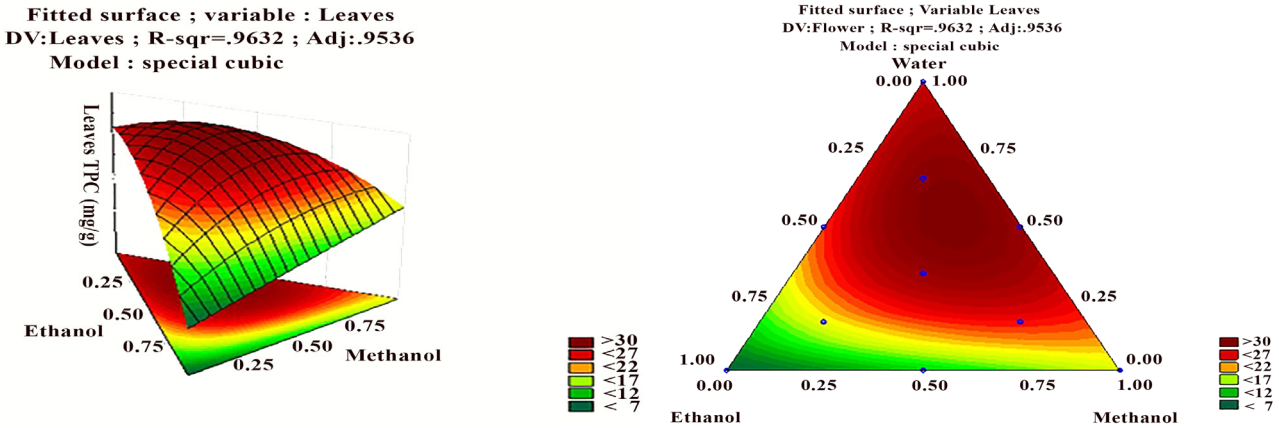


Fig. 3. Response surface contour (A: left) and response surface (B: right) plots of the special cubic model predicted TPC as a function of the ethanol, water and methanol proportions.

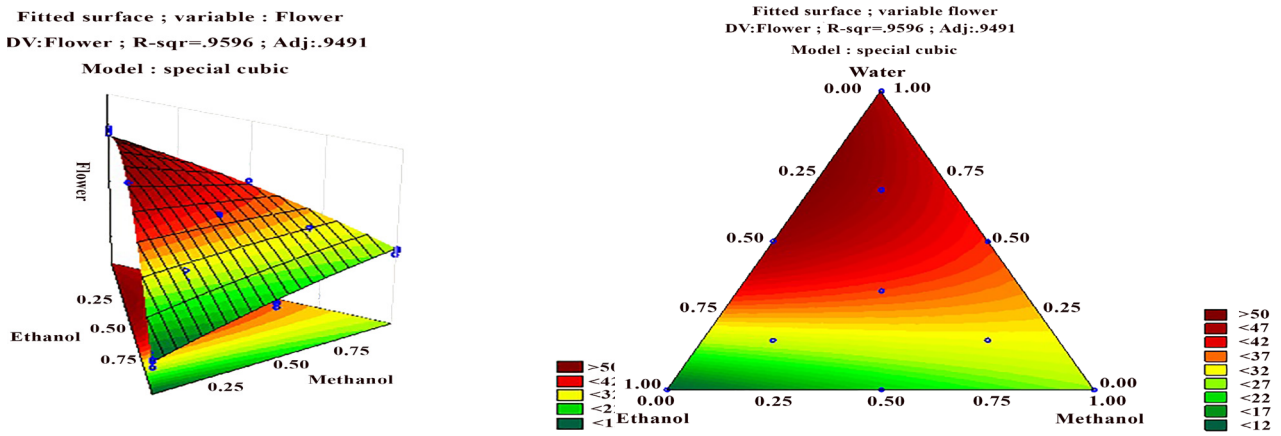


Fig. 4. Response surface contour (A: left) and response surface (B: right) plots of the special cubic model predicted TPC as a function of the ethanol, water and methanol proportions.

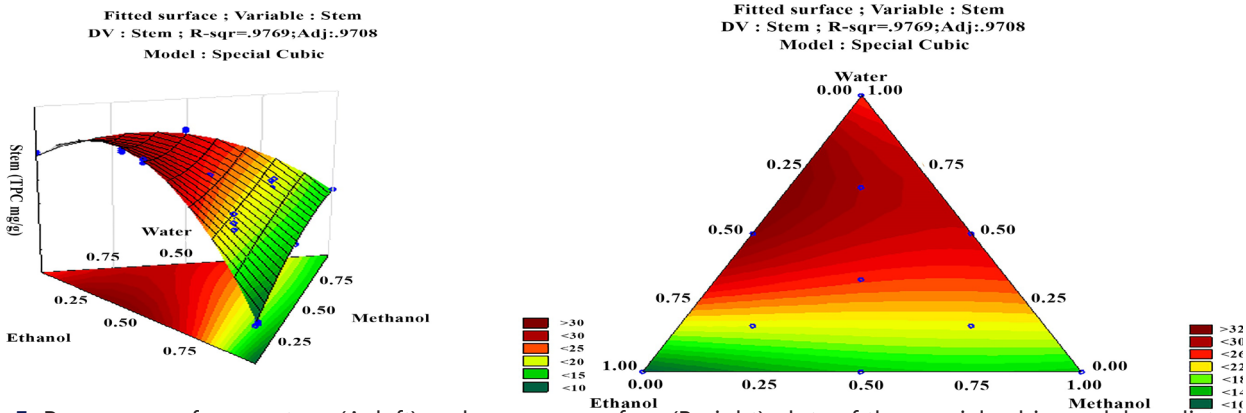


Fig. 5. Response surface contour (A: left) and response surface (B: right) plots of the special cubic model predicted TPC as a function of the ethanol, water and methanol proportions.

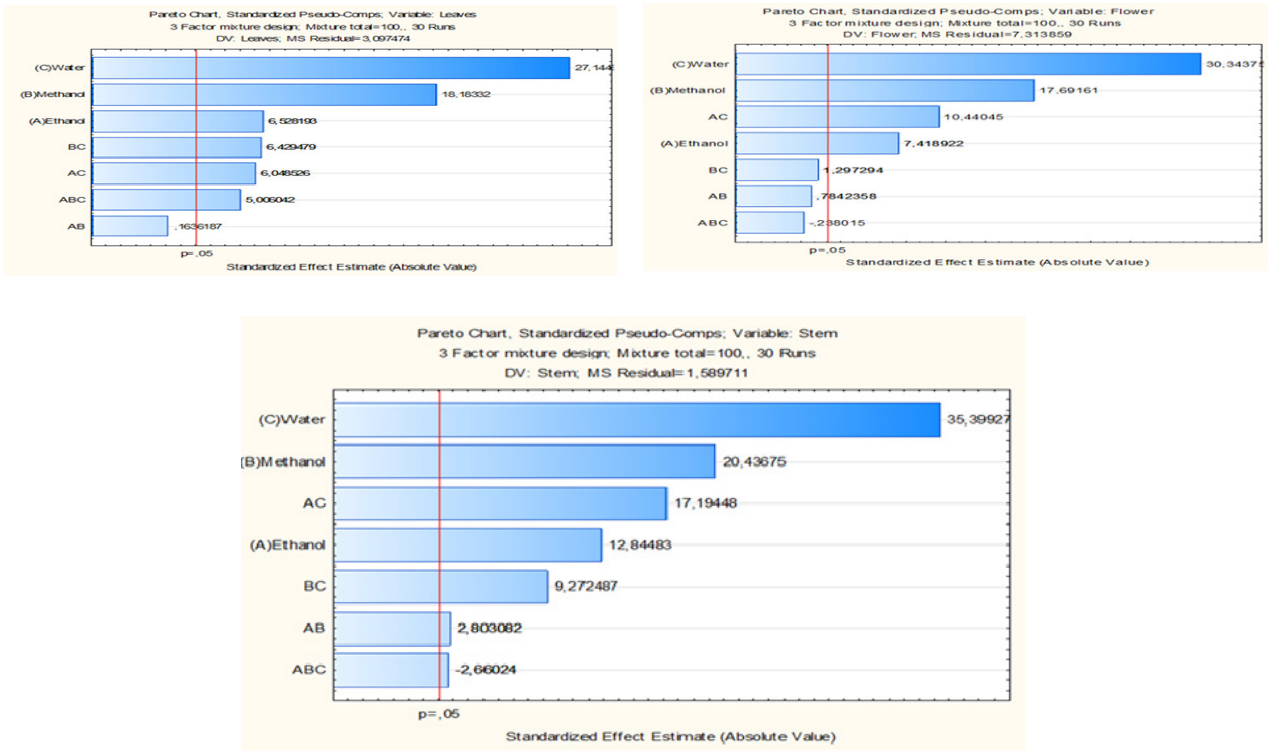


Fig. 6. Pareto charts of the standardized effects on TPC from leaves (A: Above left), flowers (Above right B), and stems (C: Below).

Table 4
The antioxidant activity of extracts of different AV parts.

	TPC mg GAE/g	TAC mg GAE/g	CUPRAC mg AAE/g	HCA mg CGAE/g
Flower	49.71±0.12	62.07±2.98	0.99±0.05	11.39±0.39
Stem	32.09±0.44a	48.63±3.69 a	0.93±0.09	4.06±0.61 a
Leaf	31.29±0.59 a	29.71±0.29 ab	1.09±0.02	4.85±0.73 a

Values in the same column followed by the same letter are not significantly different by Tukey's multiple range test ($p < 0.05$).

combination. The flower extract contained the highest TPC (49.72 ± 0.12) compared to other studied extracts. The same extract exhibited the highest antioxidant ability examined by TAC and HCA with values of 62.07 ± 2.98 mg/g GAE and 11.39 ± 0.39 mg/g CGAE, respectively.

3.5. Phenolic profile of optimized extraction of different parts of *A. visnaga*

Table 5 shows the findings of the determination of the phenolic profile of different optimized extracts of *A. visnaga* (L.), including stem, leaves and flowers. The phenolic profile was determined using HPLC to provide a scientific basis and the variability of phytochemistry of different parts of Moroccan *A. visnaga* (L.) widely used in the traditional pharmacopeia. Quantification and determination of the phenolic profiles of different parts revealed 21 compounds with different levels in the plant parts under study.

Treatment of the metabolomics profile of different parts of *A. visnaga* (L.) revealed high variability. The most prevalent phenolic compound was chlorogenic acid, which was found in varied concentrations in the flower, stem, and leaf, being 222.24, 146.34, and 16.75 µg/g, respectively. In the second rank, isorhamnetin-3-*O*-rutinoside registered the following values 130.88, 46.66, and 18.14 µg/g for flower, stem, and leaf, respectively. The levels of caffeic acid in different parts under study were 76.83, 5.67, and 18.45 µg/g, respectively. The flowers of *A. visnaga* (L.), which constituted the majority of the plant under investigation, exhibited the highest concentration of individual phenolics identified through UHPLC-DAD analysis (Table 5). These findings will be of crucial importance for further experimental investigations to determine the biological properties of *A. visnaga* (L.) as well as to enrich the Moroccan databases about phytochemicals of the plant under study.

noteworthy that the floral extract was the extract with the highest concentration of phenolic components, which are closely associated to its antioxidant properties

Table 5
Phenolic compounds of optimized extraction of different parts of *Ammi visnaga* (L.).

Phenolic compounds	Flower	Stem	Leaf
	µg/mg		
Chlorogenic acid	222.24	146.34	16.75
Isorhamnetin 3-*O*-rutinoside	130.88	46.66	18.14
Caffeic acid	76.83	5.67	18.45
Isorhamnetin 3-*O*-glucoside	71.39	41.70	15.95
Kaempferol 3-*O*-glucoside	50.95	2.72	4.74
Rutin	46.90	4.31	15.55
p-Coumaric_acid	20.51	1.89	3.34
Quercetin 3-*O*-glucoside	19.63	4.21	31.48
Isorhamnetin	19.13	1.50	11.32
Neochlorogenic acid	14.42	4.51	9.53
Kaempferol	8.91	0.32	0.91
Quercetin 3-*O*-rhamnoside	3.81	NF	0.05
Dihydroquercetin	1.47	NF	NF
Quercetin	0.20	NF	1.33
p-Hydroxybenzoic acid	0.78	0.87	1.57
Luteolin	NF	0.21	NF
Phlorizin	NF	0.02	NF
Myricetin	NF	0.08	NF
Eriodictyol	NF	0.07	NF
Naringenin	0.08	0.02	NF
Hispidulin	0.03	0.04	NF
SUM (µg/mg)	688.16	261.14	149.12

NF: Not found

(Table 4). Numerous studies have been published to assess the phytochemical profile of *A. visnaga* (L.), and they revealed that many components of the plant were identified in varying concentrations, including γ-pyrones, coumarin, khellin, visnagin, 4-norvisnagin, khellinol, visamminol, ammiol, and khellol (Hashim et al., 2014; Khalil et al., 2020). The accumulation of phenolic compounds is not distributed equally through different parts of the plants (Padda and Picha, 2007; Jia et al., 2022). Roots appear to have the lowest amounts of phenolic compounds than other parts of the plant under study (Jung et al., 2011). The aerial parts, especially leaves of *A. visnaga* (L.), contain significant quantities of phenolics than other parts of the plants, which are used to make tea due to their high radical scavenging activity (Islam et al., 2002; Oki et al., 2002; Jung et al., 2011).

Zaher et al. (2022) detected 46 individual phenolic compounds, including edulisin III, binapacryl, khellin, and visnagin, which represent 89.89% of the chemical composition of *A. visnaga* (L.). Other studies documented that the most abundant phenolics were quercetin, rhamnocitrin, rhamnetin and rhmnazin (Harborne and King, 1976; Khalil et al., 2020). The findings of this work are in high concordance with results evoked by Activity et al. (2011). In fact, *A. visnaga* (L.) is a dense source of bioactive compounds that act their positive impacts synergistically by affecting several physiological functions.

4. Concluding remarks

In the present study, optimization of the bioactive compound extraction from different aerial parts of *A. visnaga* (L.) was performed using response surface methodology. This mathematical tool increased the recovery of extracts with considerable amounts of phenolics and remarkable antioxidant activity. The special cubic model was adequate for the experimental results of specific tests. The analysis of the model-derived response surfaces revealed that in binary mixtures (50% ethanol and 50% methanol), the yielded values of phenolic compounds and the antioxidant activity increase with the water proportion of different prepared mixtures. Therefore, flowers contained the highest amounts of bioactive compounds, *e.g.*, chlorogenic acid,

isorhamnetin-3-*O*-rutinoside, caffeic acid, isorhamnetin 3-*O*-glucoside, and other compounds in remarkable quantities. Otherwise, the obtained findings constitute a primary step to recuperate the highest amount of bioactive components with considerable antioxidant activity for further experimental investigations.

Author contribution statement

Conceptualization and literature search were performed by Zineb El Jabboury, Smail Aazza and Lahsen El Ghadraoui. The first draft of the manuscript was prepared by Zineb El Jabboury and Driss Ousaaid. Uros gasic, Peda Janackovic, Zora Stevanonovic, Stefan Kolasinac performed the phytochemical profile. Oumaima Chater, Meryem Benjelloun critically analyzed and gave suggestions to finalize the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that there is no conflict of interest.

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Original Research Article

A comparative study on the extracts from the fruits of *Ficus auriculata* L.: GC-MS profiling, phytochemical composition, biological activities and *in-silico* ADMET study

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ABSTRACT

Ficus auriculata L., commonly known as, "elephant ear fig" is a species of fig tree in Moraceae family and globally found in tropical and subtropical forests. The present comparative study investigated the GC-MS analysis, phytochemical composition, *in vitro* antioxidant assays and antidiabetic activity of methanol and hexane extracts from the fruits of *Ficus auriculata* which was collected from two different agro-climatic conditions in Uttarakhand, namely Almora (Hill region) and Haldwani (Tarai region). The GC-MS analysis of Almora unripe hexane fruit extract (AUFHE) and Haldwani unripe hexane fruit extract (HUFHE) gave rise to the characterization of two chemical profiles composed of 37 and 40 bioactive compounds with γ-sitosterol (15.46% and13.44%) as the most abundant component, respectively. Moreover, in Almora unripe methanol fruit extract (AUFME) and Haldwani unripe methanol fruit extract (HUFME), 24 and 23 bioactive compounds were characterized among which linoleic acid (71.41%) and hexadecadienoate (26.42%) were the most prevalent compounds, respectively. In view of the obtained results, HUFME exhibited prominent total phenolic, flavonoid and tannin contents. AUFME also showed potent antioxidant activity when using DPPH (2,2-diphenylpicrylhydrazyl) radical scavenging activity assay (IC_{50} =447.45 ± 0.53 µg/mL), whereas strong metal chelation assay was found for HUFHE (IC_{50}= 502.07 ± 2.50 µg/mL). Furthermore, AUFME and HUFME displayed potent anti-diabetic activity. In addition, ADMET study predicted that *F. auriculata* could be considered an effective bioactive source of phytoconstituents for various biological efficacies. The observed pharmacological properties could be attributed to the presence of polyphenols, flavonoids and fatty acids in *F. auriculata* fruit.

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1. Introduction

The use of medicinal plants is widely spread all over the world. Due to the therapeutic and healing properties of the medicinal plants, they have been widely used since ancient times (Mohammadhosseini et al., 2019; Mohammadhosseini et al., 2021; Agrawal and Jain, 2023; Anish et al., 2023). In India, forests constitute multitude aromatic and medicinal plants which are used as raw materials for the production of commercial drugs and also perfumery products. As per WHO recommendation (2004), around 80% of the world's population relies on the potential use of medicinal plants and herbs. Approximately, 21,000 varieties of plants are used in a wide range of medicinal disciplines. Currently, cancer, non-communicable diseases, and mental health disorders like Alzheimer's and Parkinson's diseases are common challenges for the human health. Foodborne illnesses, which are a major health concern, are often the result of pathogenic bacteria transmitted by humans (Mehra et al., 2022). Phytonutrients such as alkaloids, anthocyanins, betacyanins, anthraquinones, coumarins, flavonoids, saponins, tannins, terpenoids, triterpenoids, glycosides, phenol, steroids, proteins, and vitamin C in herbal medicines have numerous health benefits. According to Bahl et al. (2022), plants are capable of synthesizing a vast range of secondary metabolites with therapeutic potential to cope with diseases caused by oxidative stress. *Ficus auriculata* L., a member of the Moraceae family, is known for its diverse traditional medicinal applications that set it apart from other plant species. The *Ficus* plants are primarily found in temperate, tropical, and subtropical regions at altitudes ranging from 1800 to 2600 meters. These species are native to Asia, particularly in countries such as India, Nepal, Bhutan, Pakistan, China, Malaysia, Thailand, Myanmar, and Vietnam and are also frequently used in the treatment of diarrhoea and dysentery (El-Fishawy et al., 2011).

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F. auriculata fruit is very tasty and serves as a rich source of antioxidants. In fact, diverse *Ficus* species contain high levels of polyphenolic compounds and flavonoids, which are responsible for their remarkable antidiabetic and antioxidant properties that help in the prevention and treatment of various oxidative stress-related diseases, *e.g.*, neurodegenerative and hepatic diseases (Paasyeva et al., 2020).

Various health advantages, including antimicrobial, anticancer, and anti-inflammatory properties have been linked to the majority of *Ficus* species. The impact of a methanol extract from *F. auriculata* leaves on blood clotting was examined, along with the potential antimicrobial effects of the fruits, leaves, and bark of this herbal plant (Tamta et al., 2021). The synthesized silver nanoparticles from the leaves of *F. auriculata* displayed antioxidant property (Mehra and Tandon, 2021).

In each society, a significant portion of the population is impacted by chronic inflammatory diseases. In today's society, there is a growing emphasis on health, leading many individuals to turn to herbal remedies for treating inflammation. It has been documented that *F. auriculata* fruits have anti-inflammatory and antidiabetic properties (Walia et al., 2022), suggesting that their bioactive components could be utilized in creating new culinary and medicinal products. Researchers have found that *F. auriculata* possesses various medicinal properties (Mehra and Tandon, 2021).

Nowadays, there is a significant focus on using *in silico* methods to predict the toxicity and drug likeliness of chemical compounds in the early stages of drug discovery. It is advised to assess the bioavailability of a compound before proceeding to clinical trials to avoid potential failures in the initial stages of drug development (Jia C et al., 2020). The ADMET study uses computational modeling to predict the toxicity and pharmacokinetics of a large database of compounds, both *in vivo* and *in vitro*, by improving their chemical characteristics (Davis et al., 2004). The current study aimed to assess the organic extracts of *F. auriculata* fruits collected from two distinct climatic regions in India (Almora and Haldwani). The goal was to identify the bioactive compounds in their chemical profiles using GC-MS, quantify phytoconstituents, and evaluate their antioxidant and antidiabetic properties. Additionally, an *in silico* ADMET study was conducted to predict the drug likeliness and pharmacokinetics of the bioactive compounds found in the methanol and hexane extracts of *F. auriculata*.

2. Experimental

2.1. Materials and methods

The unripe fruits of *F. auriculata* L. were collected from two different altitudes of Uttarakhand, namely Almora (latitude-N 29° 30.89652′ and longitude-E 79° 59.9289′) and Haldwani (latitude-N 29° 13.09584′ and longitude-E 79° 30.77862′). The collection was carried out in the months of June and July and the collected samples were identified by a plant taxonomist (Dr. D.S. Rawat) from Department of Biological Science, Govind Ballabh Pant University of Agriculture and Technology, India.

2.1.1. Drying and extraction

After sampling, the unripe fruits of *F. auriculata* were washed thoroughly with water to get rid of dust and other impurities. After washing off, the fruits were chopped into small pieces and left to dry at room temperature for two weeks. The moisture content in the plant materials was removed and the dried samples were grounded to fine powder. The powder material was extracted with successive soxhlet method using methanol and hexane solvents, respectively.

2.1.2. Soxhlet extraction

The unripe fruits of *F. auriculata* were first processed in a grinder to get a powder of fine and homogenous size. The thimble of filter paper was first filled with a powder and then placed inside the soxhlet apparatus, where the extraction was carried out using successive portions of hexane and methanol. The temperature was kept constant at 60 °C, and each extraction lasted about 9-10 hours. Then, the powder from the thimble was utilized for the successive extraction using methanol after the hexane extract had been collected. The same procedure has been followed for methanol. The obtained extracts were subsequently evaporated in a water bath at 40 °C, dried in a vacuum oven at 40 °C, and the yield value(%) was then determined for each extract. Then, the dried extracts were subjected to phytochemical screening and biological activity.

2.1.3. GC-MS analysis

The phytoconstituents present in the hexane and methanol extracts of *F. auriculata* fruits were identified by GC-MS analysis. Gas chromatography-mass spectrometery was performed at the Advanced Instrumental Research Facility, Jawahar Lal Nehru University (JNU), New Delhi. In this relation, *F. auriculata* unripe fruits were analyzed for their chemical constituents using a combined gas chromatography (GC) HP 6890 with mass selective detector MS 5973 equipped with a split-splitless injector, an electronic pressure control, and a DB-5 silica column (30 m X 0.25 µm film thickness) (Agilent Technologies, USA). Helium was used as a carrier gas at the flow rate of 1.0 mL min^{-1}. The injector was operated at 250 °C and the oven temperature was programmed at 60 °C for 15 minutes. The detection was performed in full scan mode over the *m/z* range of 41-450. The Calibur 4.0 software was used to analyze the data, and the chromatograms were identified by matching their mass spectral fragmentation patterns which were compared to the database stored at the National Institute of Standards and Technology (NIST 2.2) library.

2.2. Quantitative phytochemical analysis

2.2.1. Determination of total phenolic content (TPC)

The total phenolic content of the methanol and hexane

extracts from the fruits of *F. auriculata* was assessed using the standard procedure given by Orphanides et al. (2013). In accordance with this method, in each test tube, 5 mL of FCR (Folin-Ciocalteu Reagent, 1 N) was added to 0.5 mL of each hexane and methanol extract of *F. auriculata* fruit. 1 mL of saturated sodium carbonate solution (1 N) was then added to the reaction mixture to neutralize it. The reaction mixture was kept at 24 °C for 35 minutes. In the next step, the reaction mixture was centrifuged at 4000 rpm for 10 minutes. Then, the absorbance was measured against the reagent blank at 725 nm. The total phenolic content (TPC) was finally quantified using a standard curve of gallic acid at different concentrations (30-150 µg) and results were recorded in terms of mg gallic acid equivalents (mg $GAEg^{-1}$).

2.2.2. Determination of total flavanoid content (TFC)

The standard procedure for the determination of total flavonoid contents was according to Orphanides et al. (2013) report. Briefly, 4 mL of distilled water and 300 µL of $NaNO_2$ (5.0%) solution were added to 1 mL of each hexane and methanol extract from the fruits of *F. auriculata*. After mixing, the solution was incubated for 5 minutes followed by the addition of 300 µL of $AlCl_3$ (10%) to the reaction medium which was allowed to stand for 1 minute. Immediately after, 2 mL of NaOH (1 M) was added to the reaction mixture followed by 2.4 mL of distilled water. All the reagents are mixed and incubated in a dark place for 15 minutes at 24 °C, centrifuged at 4000 rpm for 5 minutes and the relevant absorbance was measured at 510 while using catechin as the standard. Finally, total flavonoid content was expressed in terms of mg $CAEg^{-1}$.

2.2.3. Determination of proanthocyanidin

The evaluation of proanthocyanidin was done according to a standard method developed by Sun et al. (1998). Accordingly, 3 mL of methanol solution of vanillin was added to 1 mL of each hexane and methanol fruit extract of *F. auriculata* in a test tube followed by 1 mL of HCl. The reaction mixture was then incubated for 15 minutes and the corresponding absorbance was recorded at 500 nm while using catechin as a standard. Finally, proanthocyanidin content was expressed in terms of mg $CAEg^{-1}$.

2.2.4. Determination of orthodihydric phenol

The orthodihydric phenol content of the prepared extracts was assessed by a standard method (Mahadevan et al., 1986). In this context, hexane and methanol extracts of *F. auriculata were first* mixed with 1 mL of HCl (0.5 N) and after that, 1 mL of arnows reagent consisting of 10 g HNO_3 + 10 g of sodium molybdate dissolved in 100 mL of distilled water was added to the reaction medium. After the addition of 2 mL of NaOH (1 N) to the reaction mixture, 4.5 mL of distilled water was added. Thereafter, at 515 nm, the relevant absorbance was recorded against a reagent blank using catechol as a standard and orthodihydric phenol was expressed in terms of mg $CLEg^{-1}$.

2.2.5. Determination of total tannin

The total tannins of *F. auriculata organic extracts* were determined according to the method given by Sadasivam et al. (1992). In this regard, 0.5 mL of Folin-Denis reagent was mixed with 1 mL of each hexane and methanol extract of *F. auriculata* and 6.6 mL of distilled water. Thereafter, 1 mL of saturated sodium carbonate solution was added followed by the addition of distilled water. The reaction mixture was kept at room temperature for 30 minutes and the absorbance was measured against a reagent blank at 700 nm. Tannic acid was used as a standard and the total tannins were reported as mg tannic acid equivalents (mg TAE^{-1}).

2.3. Biological study

2.3.1. Antioxidant activitiy

2.3.1.1. DPPH radical scavenging activity

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of the fruit extracts of *F. auriculata* was monitored using a previously reported procedure (Chen et al., 1995). Antioxidant activity is based on the potential of an extract to scavenge the free DPPH radicals present in the reaction medium. To determine the potential antioxidant activity of the organic extracts, 1 mL of each hexane and methanol extract of *F. auriculata* was incubated in the dark at room temperature with 5 mL of a DPPH solution (0.4 mM) for 30 minutes. The reaction mixture's absorbance was measured at 517 nm using ascorbic acid and gallic acid as standards, and a blank solution as the control.

2.3.1.2. Metal chelating method

The metal chelating behavior of the prepared organic extracts from the fruits of *F. auriculata* was monitored regarding the method developed by Hsu et al. (2003) on the basis of the chelating ability of Fe^{2+} ions of antioxidants. Accordingly, 1 mL of each hexane and methanol extract of *F. auriculata* was treated with 0.1 mL of $FeCl_3.6H_2O$ (Iron (III) chloride hexahydrate, 2 mM) reagent, followed by 0.2 mL of ferrozine solution (5 mM). A test sample with different concentrations (200-1000 µg/mL) was taken. EDTA (Ethylene diamine tetraacetate) was used as a standard and the reaction mixture was shaken vigorously and incubated at room temperature for 10 minutes. The absorbance was measured at 562 nm and Fe^{2+} chelating activity was compared with the concentration of standards.

2.4. *In vitro* antidiabetic activity

One of the goals of the current investigation was to provide the potential inhibitory evidence for *F.*

auriculata fruit extracts on alpha-amylase and alpha-glucosidase enzymes in accordance with the method proposed by Kidane et al. (2018).

2.5. Alpha-amylase inhibitory activity

The methanol and hexane extracts of *F. auriculata* having concentrations over the range 200-1000 µg/mL were placed in a test tube. A sodium phosphate buffer (0.02 M, pH-6.9) solution was added to the alpha-amylase enzyme. The reaction mixture was incubated at 25 °C for 10 minutes. A starch solution (1.0%) was then added to the sodium phosphate buffer (0.02 M). The reaction mixture was incubated again for 10 minutes at 25 °C followed by the addition of 2 mL of DNS (3,5-dinitrosalicylic acid, 40 mM) reagent for the completeness of the reaction. The mixture was finally incubated for 5 minutes in a water bath at 35 °C and subsequently cooled at room temperature. In the final step, the mixture was diluted with distilled water and the relevant absorbance was taken at 540 nm against a reagent blank while using acarbose as a standard.

2.6. Alpha-glucosidase inhibitory activity

The procedure of alpha-glucosidase inhibitory activity was initiated by incubating maltose substrate (6 mM) with 1 mL of tris buffer (pH 8.0) and various concentrations of each hexane and methanol extract from the fruits of *F. auriculata* (200-1000 µg/mL) at 35 °C. Alpha-glucosidase enzyme was added into the reaction mixture to initiate the reaction. To stop the reaction, DNS color reagent was introduced. The intensity of the color was measured by assessing the absorbance at 540 nm.

2.7. *In-silico* ADMET study

To evaluate the *in silico* ADMET study, structure of the selected compounds were identified via GC-MS and drawn using Chem draw. Firstly, the selected compounds were transformed into canonical SMILES format and their drug-like and pharmacokinetic characteristics were forecasted using ADME tool by a Swiss ADME online web server, according to the developed protocol (Daina et al., 2017). The toxicity profiling was done using ProTox-II webserver. The toxicity of the selected compounds was forecasted in the sense of different criteria, *i.e.*, oral toxicity, organ toxicity (Hepatotoxicity) and toxicological end points (Mutagenicity, cytotoxicity, immunotoxicity and carcinotoxicity).

2.8. Statistical analysis

Correlation and PCA were performed using R Studio (Version 4.2.2, 2022-10-31) in Intel^{R} Core (TM), i5-103 5GI CPU @1.00Ghz on 64 bit windows with 8 GB RAM.

3. Results and Discussion

3.1. Percent yield

The yields of the hexane and methanol extracts from the fruits of *F. auriculata* have been presented in Table 1. As can be seen in this table, the highest yield has been obtained for the methanol extract of the AUFME sample, whereas the lower yield has been found for the hexane extract of the AUFHE sample.

3.2. GC-MS analysis

The peak areas (%) of the bioactive compounds in the hexane and methanol extracts from the fruits of *F. auriculata* using GC-MS have been shown in Table 2. As seen, a wide the range of compounds have been characterized which demonstrate significant antioxidant, antidiabetic, and various pharmacological effects.

In total, 23 phytoconstituents were observed in HUFME, accounting for 97.94% of the overall peak areas of the chemical profile comprising hexadecadienoate (26.42%), γ-sitosterol (15.57%), linoleic acid (methyl ester) (7.17%), palmitic acid (methyl ester) (5.45%) as the most abundant constituents. In AUFME, a total of 24 phytoconstituents were identified constituting 99.82% of total composition with linoleic acid (71.41%), quinic acid (kinic acid) (10.61%), and γ-sitosterol (3.33%) as the major bioactive components. In the hexane extract of HUFHE, a total of 40 phytoconstituents were identified accounting for 93.98% of the total composition and the major identified bioactive components were found to be γ-sitosterol (13.4%), lupeol (9.26%), oleic acid (methyl ester) (9.71%), whereas in AUFHE, a total of 37 phytoconstituents were identified altogether making up 97.09% of hexane extract composition and the major characterized bioactive components were β-sitosterol (15.46%), lupeol (10.36%), octacosanol (9.11%) and petroselinic acid (methyl ester) (6.64%).

The result obtained reveals that AUFME was characterized by high levels of fatty acids in decreasing order of ketones, aldehydes, esters, and alcohols, whereas phytosterols and terpenoids in AUFHE have been detected at high levels. In HUFME, fatty acids and phytosterol have been detected at high levels, whereas in HUFHE, phytosterol, fatty acids and terpenoids have been detected at high levels. Visual interpretation of GC-MS depicted metabolite profiles of both fractions varied quantitatively, and these tentative compounds were compared with the reported literature and databases. A previous study depicted that *Ficus* spp. viz. *F. auriculata* fruits have organic acids, *e.g.*, citric and quinic acids (Boelsma et al., 2001). On the other hand, stearic, oleic, linoleic, and linolenic acids were reported. In addition, steroids were identified, *i.e.*, stigmasterol in the ethanol extract of *F. auriculata*. Chemical compounds were also reported in a previous study comprising quercetin, isoquercetin, quercitrin, kaempferol, catechin, epicatechin, myricetin, vitexin, apigenin, rutin, gallic acid, vanillic acid, chlorogenic acid, and caffeic acid (Yunus et al., 2021).

Flavanols, flavanonols, flavones, flavanones, flavonolignans, anthocyanins, hydroxycinnamic acids, and derivatives were also characterized in some *Ficus* species (Elhawary et al., 2018). However, the present study revealed that the fruit extracts of *F. auriculata*

Table 1
Percent yields of hexane and methanol extracts of of *F. auriculata* fruits.

Solvent	Sample	Weight(g)	Yield(g)	Yield (%
Methanol	HUFME	206	11.303	5.48
	AUFME	222	15.28	6.88
Hexane	HUFHE	220	8.46	3.84
	AUFHE	230	5.97	2.59

HUFME-Haldwani Unripe Fruits Methanol Extract, AUFME-Almora Unripe Fruits Methanol Extract, HUFHE-Haldwani Unripe Fruits Hexane Extract AUFHE-Almora Unripe Fruits Hexane Extract.

Table 2
GC-MS analysis of hexane and methanol extracts from the fruits of *F. auriculata*.

Compounds	% Composition			
	HUFME	AUFME	HUFHE	AUFHE
Benzenedicarboxylic acid, (dioctyl ester	0.43	-	-	-
Cetane	0.81	0.93	0.32	-
Cyclopentyl propionic acid	0.65	-	-	-
Cyclopentylpropionic acid	3.59	-	-	-
Docosanoic acid(methyl ester)	0.37	-	-	-
Dodecane	3.65	0.28	-	-
Hexadecadienoate	26.42	-	-	-
Hexyloxacyclotridec-10-en-2-One	0.89	-	-	-
Hydrocinnamic acid,	1.56	-	-	-
Linoleic acid (methyl ester)	7.17	-	-	1.7
Lupeol	5.85	-	9.26	10.36
Methyl commate B	2.58	0.44	-	-
Palmitic acid(methyl ester)	5.45	0.65	2.07	1.57
Palmitoyl glycerol	1.97	-	-	
Stearic acid(methyl ester)	2.47	-	-	0.48
Stigmastenol	1	0.4	-	-
Stigmastenone	2.33	-	-	-
Stigmasterol	2.73	-	3.17	-
Tetradecane	3.52	0.57	-	-
y-sitosterol	15.57	3.33	13.44	-
α-Linolenic acid	0.79	-	-	-
α-Tocopherol	2.75	0.14	-	-
β-Amyrone	5.39		-	-
(-)-Spathulenol	-	0.12	-	-
Aromadendrane	-	0.19	-	-
Decyl acetate	-	0.19	-	-
Eicosatrienoic acid(methyl ester)	-	4.9	-	-
Ergostadienol	-	0.25	-	-
Methyl commate D	-	0.72	-	-
Neophytadiene	-	0.16	-	0.22
Octadecadienoic acid	-	71.41	-	-
Octadecane	-	0.78	-	-
Palmitic amide	-	0.25	-	-
Pluchidiol	-	0.95	-	-

Table 2 Contined

Compounds	Composition (%)			
	HUFME	AUFME	HUFHE	AUFHE
Quinic acid	-	10.61	-	-
Squalene	-	0.15	5.89	1.7
y-Gurjunenepoxide	-	0.29	-	-
β-Amyrin acetate	-	0.22	-	-
Icosane	-	-	0.3	-
Juniper camphor	-	-	0.25	-
1-Pentadecanol	-	-	0.34	-
1-Hexadecanol	-	-	0.43	-
Butylated hydroxytoluene	-	-	0.28	0.25
Ethyl tridecanoate	-	-	1.29	-
Hexadecadienoic acid, methyl ester	-	-	2.23	-
Oleic acid, methyl ester	-	-	9.71	-
Methyl stearate	-	-	0.65	-
Linolelaidic acid	-	-	1.01	-
Ethyl oleate	-	-	1.67	-
Nonane	-	-	0.24	-
1-Octadecene	-	-	0.93	-
Octadecyl 2-propyl ester	-	-	0.36	-
4,8,12,16-Tetramethylheptadecan-4-olide	-	-	0.47	-
Ethyl linalool	-	-	1.15	-
Oxalic acid	-	-	0.33	-
9-Tricosene	-	-	2.33	2.96
2-Methyl-1-hexadecanol	-	-	0.45	-
Di-N-octyl phthalate	-	-	0.38	-
1-Heneicosyl formate	-	-	1.16	-
Geranyl linalool	-	-	0.83	-
Cyclobutylcarboxylic acid	-	-	1.09	-
Amyrin, acetate	-	-	6.69	-
Lupenyl acetate	-	-	2.09	4.94
Lupenone	-	-	1.73	1.67
Nonacos-1-ene	-	-	4.11	-
Cholesterol	-	-	0.86	-
Campesterol	-	-	4.43	1.04
Sitostenone	-	-	4.73	1.35
Arachidic alcohol	-	-	7.31	-
3-Hexadecene	-	-	-	0.57
Phenol	-	-	-	3.3
Pentadecene	-	-	-	1.15
Nonadecane	-	-	-	0.23
Heptadecene	-	-	-	2
Phytone	-	-	-	0.23
O-Benzoquinone	-	-	-	0.19
Nonadecene	-	-	-	1.25
Petroselinic acid (methyl ester)	-	-	-	6.64

Table 2 Contined

Compounds	Composition (%)			
	HUFME	AUFME	HUFHE	AUFHE
Linoleic acid	-	-	-	0.26
Oleic acid (ethyl ester)	-	-	-	0.16
Docosanol	-	-	-	0.64
Heptacosane	-	-	-	0.69
Hexadecanol	-	-	-	0.2
Dioctyl phthalate	-	-	-	0.39
Heptacosene	-	-	-	1.62
Octacosane	-	-	-	2.48
Celidoniol	-	-	-	0.63
Dodecyl tiglate	-	-	-	0.99
Nonacosene	-	-	-	4.57
Octacosanol	-	-	-	9.11
Pentatriacontene	-	-	-	7.79
α-Tocopherol	-	-	-	1.86
β-Amyrin Acetate	-	-	-	6.44
β-Sitosterol	-	-	-	15.46

contain considerable amounts of both saturated (palmitic acid) and unsaturated fatty acids, *e.g.*, linoleic acid, oleic acid, and hexadecadienoate and lower levels of the others. AUFME is rich in unsaturated fatty acids. Saturated fats play a crucial role in cardiovascular health and play an important role in the appropriate release of insulin (Farhana et al., 2022). Moreover, unsaturated fatty acids reduce the risk of diabetes. Hexadecadienoate has antioxidant and other pharmacological roles (Dubey et al., 2020). It was previously reported that quinic acid belongs to the cyclitol class found in AUFME and that it is the major constituent of coffee and a strong antioxidant with many beneficial effects (Ma et al., 2015). Petroselinic acid plays a significant role in the food, chemical, and cosmetics industries (Uitterhaegen et al., 2016). Fatty alcohols (octacosanol) are found in AUFHE as the major constituting group. Sharma et al. (2020) revealed that lupeol has various pharmacological responses, including decreasing the levels of calcium-oxalate and having cytoprotective action against free-radical-induced damage. Phytosterols like γ-sitosterol and β-sitosterol have been characterized in AUFHE and HUFHE samples, respectively. A wide number of studies have reported remarkable pharmacological effects. β-Sitosterol, a well-known phytosterol, was used as sunscreen emulsion and anti-ageing additives in cosmetic products (Dweck, 2006). It has previously been reported that γ-sitosterol could be used in the development of protein anti-diabetic drugs (Tripathi et al., 2013). Almora fruits of *F. auriculata* contain appreciable and comparable amounts of saturated and unsaturated fatty acids, terpenoids, phytosterols, and other bioactive compounds that are found in trace quantities as compared with Haldwani fruits justifing the traditional uses of this important medicinal plant for the treatment of various diseases. Molecular docking of the identified major phytoconstituents with their receptors can be carried out in the future, which can show further potential pharmacological activities like antidiabetic, anti-inflammatory, anticancer, etc.

3.3. Quantitative phytochemical analysis

The results of the phytochemical screening of the fruit organic extracts of *F. auriculata* have been represented in Table 3. As can be seen in this table, the highest quantities of TPC, TFC, OPC, proanthocynidin and tannin were found in the methanol extract of HUFME sample. However, the least values for the TPC, TFC and OPC were observed for the hexane extract of AUFHE. The hexane extract of HUFHE sample also showed the lowest proanthocynidin and tannin contents.

3.3.1. Total phenolic contents

In the methanol extract from the fruits of *F. auriculata*, the maximum amount of total phenol content was examined in HUFME (41.95 ± 4.2 mg GAE/g) followed by AUFME (38.99 ± 2.24 mg GAE/g). Total phenolic content found in HUFHE (28.19 ± 2.82 mg GAE/g) followed by AUFHE (25.10 ± 0.14 mg GAE/g) in the hexane extract. A previous study reported that total phenol content in *Parkia roxburghii* fruits was found to be 4.13 ± 0.52 mg GAE/g which was collected from Sikkim (Pandey et al., 2018). A previous research also found that an ethanol extract of *F. auriculata* fruits contained 33.25 ± 0.94 mg

Table 3
Phytochemical composition in methanol and hexane extracts from the fruits of *F. auriculata*.

Process Co-products	TPC (mg GAE/g)	TFC (mg GAE/g)	OPC (mg CLE/g)	Proanthocynidin (mg CAE/g)	Tannin (mg TAE/g)
HUFME	41.9±4.20	23.08±0.09	50.56±2.14	27.04±0.12	8.03±0.021
AUFME	38.99±2.24	19.6±0.22	41.75±0.531	23.17±0.056	7.71±0.048
HUFHE	28.19±2.82	14.85±0.019	29±0.87	15.11±0.14	5.44±0.059
AUFHE	25.06±0.14	13.4±0.12	22.72±2.00	15.9±0.100	6.00±0.021

TP = Total phenolic content, OPC= Orthodihydric phenol cotent, GAE/g = Equivalent of gallic acid per gram, CLE/g: Equivalent of catechol, CAE= Equivalent of catechin and TAE = Equivalent of tannic acid.

GAE/g DF (Shahinuzzaman et al., 2021). The findings of the current study show more positive results in terms of phenolic content compared to earlier studies.

3.3.2. Total flavonoid content

The highest amount of total flavonoid content in the methanol extract from the fruits of *F. auriculata* was found in HUFME (23.08 ± 0.09 mg GAE/g) followed by AUFME (19.65 ± 0.224 mg GAE/g). In the hexane extract of *F. auriculata* fruits, the highest amount of total flavonoid content was obtained in HUFHE (14.85 ± 0.019 mg GAE/g) followed by AUFHE (13.4 ± 0.128 mg GAE/g).

3.3.3. Orthodihydric phenol content

In the methanol extract of *F. auriculata* fruits, the highest orthodihydric phenol content was found in HUFME (50.56 ± 2.14 mg CLE/g) followed by AUFME (41.75 ± 0.531 mg CLE/g). However, in the hexane extract of *F. auriculata* fruits, the highest amount was obtained in HUFHE (29 ± 0.87mg CLE/g) followed by AUFHE (22.72 ± 2.00 mg CLE/g).

3.3.4. Proanthocyanidin content

In the methanol extract of *F. auriculata* fruits, the highest proanthocyanidin content was found in HUFME (27.04 ± 0.12 mgCAE/g), followed by AUFME (23.17 ± 0.056 mgCAE/g). The highest amount of CAE/g was obtained in AUFHE (15.9 ± 0.100 mg CAE/g), followed by HUFHE (15.11 ± 0.14 mg CAE/g). In the current study, quantitative determination of proanthocyanidin content has been reported for the first time for *F. auriculata*. These results indicate that proanthocyanidin is a safe and a valuable natural antioxidant.

3.3.5. Tannin content

In the methanol extract of *F. auriculata* fruits, the highest tannin content was observed for HUFME (8.03 ± 0.021 mg TAE/g) followed by AUFME (7.71 ± 0.048 mg TAE/g). The hexane extract of *F. auriculata* fruits contained the most significant tannin content in AUFHE (6.00 ± 0.021 mg TAE/g), followed by HUFHE (5.44 ± 0.059 mg TAE/g). Among all the five biochemical parameters analyzed for the methanol and hexane extracts from the fruits of *F. auriculata* from Almora (Hill region) and Haldwani (Tarai region), the methanol extract was found to have the highest orthodihydric and phenolic content compared to the hexane extract. The phenolic contents were found to be moderate followed by proanthocyanidin, flavonoid and tannins. In the present study, variation in phytoconstituents could be strongly related to abiotic factors such as the climate, geographical factors, and altitude and soil type.

3.4. Biological activity

3.4.1. Antioxidant activity

The DPPH and metal chelation radical scavenging activity of the methanol extracts of *F. auriculata* fruits from two different regions, namely Almora and Haldwani, was dose-dependent at different concentrations (200-1000 g/mL). The IC_{50} values of the methanol and hexane extracts of *F. auriculata* fruits are presented in Table 4, Fig. 1 and Fig. 2.

3.4.1.1. DPPH radical scavenging activity

In the methanol extract from the fruits of *F. auriculata*, AUFME showed strong radical scavenging activity (IC_{50} = 447.45 ± 0.53 µg/mL) followed by HUFME (IC_{50} = 465.63 ± 0.56 µg/mL) compared with the standard. In the hexane extract, HUFHE exhibits strong radical scavenging activity (IC_{50} = 597.12 ± 1.66 µg/mL) followed by AUFHE ((IC_{50} = 627.07 ± 1.08 µg/mL). A previous research depicted that ethanol extract of *F. auriculata* fruit has a DPPH radical scavenging inhibition (85.20 ± 0.96%) (Shahinuzzaman et al., 2021). However, El-Fishawy et al. (2011) reported that the ethanolic fruit extract exhibited various radical scavenging activity (44.90-88.24%) over the concentration range of 2-8 mg/mL. The reported variation in the previous studies may be due to drying methods affecting the fruits' pharmacological properties (Bushra et al., 2012), as obtained from this study. In the present study, AUFME exhibited greater maximum DPPH radical scavenging activity than HUFME. As it was investigated that phenolic content was found to be maximum in HUFME, a similar finding was also reported in the extracts of a number of medicinal plants and no correlation was observed in total phenol content and antioxidant capacity (Yilmaz et al., 2009). HUFHE was found to have maximum DPPH

radical scavenging potential as compared with AUFHE due to the presence of high levels of phytochemicals. We concluded that methanol extract of Almora unripe fruits has maximum antioxidant capacity due to its maximum orthodihydric phenolic compounds. The other reason might be the presence of other bioactive compounds like fatty acids, phytosterols, etc. The results also presented the remarkable antioxidant features and polyphenolic content which make this wild fruit a better candidate for its use in various functional foods and nutraceuticals.

3.4.1.2. Metal chelating activity

In the hexane extract from the fruits of *F. auriculata*, HUFME exhibited strong metal chelating activity (IC_{50} = 505.05±3.98 µg/mL), followed by AUFME (IC_{50} = 529.48 ± 0.736 µg/mL), while AUFHE exhibited strong metal chelating activity (IC_{50} = 502.07 ± 2.50 µg/mL), followed by HUFME (IC_{50} = 522.27 ± 1.29 g/mL). The hexane extract shows greater metal chelation potential than the methanol extract. In the hexane extract of Haldwani fruits, phytoconstituents were negatively correlated with metal chelation antioxidant activity because methanol extract contains the maximum levels of phenolic compounds. The chelating capacity of the investigated extracts from *F. auriculata* fruits decreased with decreasing polarity. According to the previous data, *F. maclellandii* and *F. racemosa* ethanolic fruit extracts had comparatively lower chelating power than *F. auriculata* (Tamuly et al., 2015). It suggested that methanol extract had potent chelating power and the methanol fraction was observed to exhibit a significant capacity to chelate ferrous ions in comparison to hexane fractions. The current outline demonstrates that chelation therapy used in formulation of synthetic compounds may contain some side effects. Thus, chelation of metal ions by natural phytoconstituents in the fruits of *F. auriculata* is of therapeutic importance.

3.4.2. Antidiabetic activity

Antidiabetic potential of methanol and hexane extracts from the fruits of *F. auriculat*, in terms of alpha-amylase and alpha-glucosidase inhibitory action, was presented in Table 5.

3.4.2.1. Alpha-amylase inhibitory activity

The highest α-amylase inhibitory action was found in AUFME (IC_{50} = 240.45 ± 1.26 µg/mL) followed by HUFME (IC_{50} = 255.1 ± 1.02 µg/mL). Anjum et al. (2019) observed that the methanol fraction of *F. auriculata* fruits recorded the maximum α-amylase. *F. auriculata* fruits might be useful in treatment of Type II Diabetes mellitus. In the hexane extract, the maximum inhibitory effect was shown in AUFHE (IC_{50} = 271.4 ± 0.84 µg/mL) followed by HUFHE (IC_{50} = 290.73 ± 0.546 µg/mL). Acarbose which is widely used as an anti-diabetic remedy, showed an IC_{50} of 230.16 ± 0.032 µg/mL. The present study highlights that less significant differences were observable between IC_{50} values of all extracts which are single-fold less potent and closer to the inhibition effect of acarbose. Such α-amylase inhibitors are also known as starch blockers as they prevent or slow the digestion of starch in the body, basically by blocking the hydrolysis of 1,4-glycosidic linkages of starch and other oligosaccharide sugars (Kumar et al., 2010). AUFME exhibited the maximum inhibitory effect. Linoleic acid, which is abundant in AUFME, has anti-diabetic activity by inhibiting protein tyrosine phosphatase linked to insulin resistance.

3.4.2.2. Alpha-glucosidase inhibitory activity

The highest alpha glucosidase inhibitory action of the prepared organic extracts from the fruits of *F. auriculata* was found in HUFME (IC_{50} = 245.02 ± 1.81 µg/mL) followed by AUFME (IC_{50} = 257.5 ± 1.38 µg/mL). Anjum et al. (2019) observed that methanol fraction of *F. auriculata* fruit reported the maximum α-glucosidase with an IC_{50} value 103.43 ± 0.67 g/mL, respectively. In the hexane extract, the maximum inhibitory effect was shown in AUFHE (IC_{50} = 291.87 ± 1.49 µg/mL) followed by HUFHE (IC_{50} = 316.7 ± 1.60 µg/mL). γ-Sitosterol and linoleic acid were responsible for antidiabetic activity (Tripathi et al., 2014). In the hexane extract of AUFHE, significant tannin content was found as compared with HUFHE. Polyphenols in *Ficus* contribute to the health benefits because of their antioxidant, antitumor, and antidiabetic efficacy (Shin et al., 2014) and inhibited α-amylase and α-glucosidase activity but less potent than methanol extract of fruits.

Further, the highest inhibitory activity shown by the methanol fraction of *F. auriculata* fruit was comparable to that of acarbose that may be attributed to the higher concentration of these compounds in the methanol fraction as compared to other fractions. Overall, correlation was observed and indicated the maximum number of phytochemicals and other bioactive compounds contribute to higher antioxidant and antidiabetic activity.

3.5. *In silico* ADMET analysis

The ADME (absorption, distribution, metabolism and excretion) attributes, pharmacokinetics and drug likeness of some chosen compounds are assessed using Swiss ADME online software presented in Table 6. As per rule, the molecular weight (MW) is < 500, topological surface area (TPSA) < 140, number of H-bond donors (nOHD) ≤ 5, H bond acceptors (nOHA) ≤ 5, rotatable bonds (nRB) ≤ 10, water partition coefficient (WLOGP) ≤ 15. The present analysis depicts that 6 out of 13 compounds exhibit potent drug like properties subsequently as per Egan's Lipinski's and Verber's rule. The bioavailability score in chosen compounds were observed 0.55 which predicts the effective bioactivity of compound. TPSA value less than 30 $Å^2$, predicts the good brain barrier potential. Effective bioactive nature was confirmed by bioavailability score 0.55 observed in all the compounds. P-glycoprotein (P-gp) substrate was not found in any molecule, recommending the fine intestinal absorption of compounds. The consensus Log Po/w observed in the range 3.26-13.34 for the compound exhibits good lipophilicity character. Palmitic

Table 4
DPPH and metal chelation IC50 value in methanol and hexane extracts from the fruits of *F. auriculata*.

Process Co-products	DPPH scavenging IC_{50} Mean ± SD (µg/mL)	Metal chelation IC_{50} Mean ± SD (µg/mL)
HUFME	465.6±0.560	505.05±3.98
AUFME	447.54±0.53	529.48±0.736
HUFHE	597.12±1.66	502.07±2.50
AUFHE	627.07±1.08	522.27±1.29
Ascorbic acid	193.9±1.12	-
Gallic acid	199.32±1.97	-
EDTA	-	197.3±1.04

*= Standard antioxidant, '-'= Not present, values are means of three replicates±standard deviation.

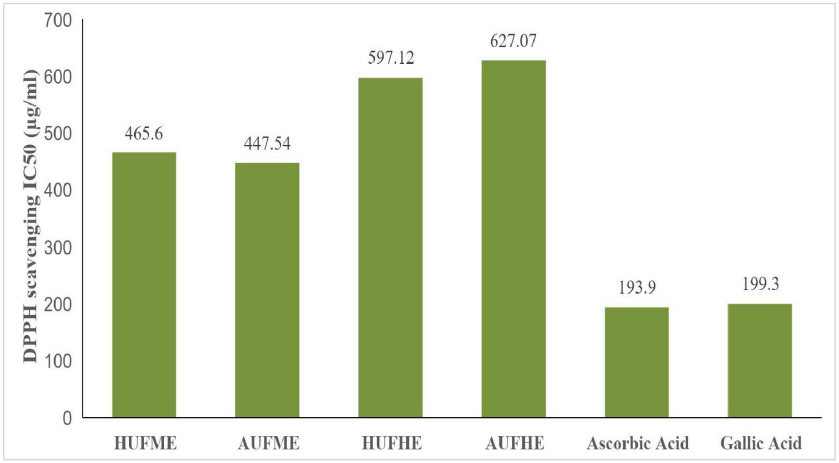

Fig. 1. DPPH activity in extracts and standards.

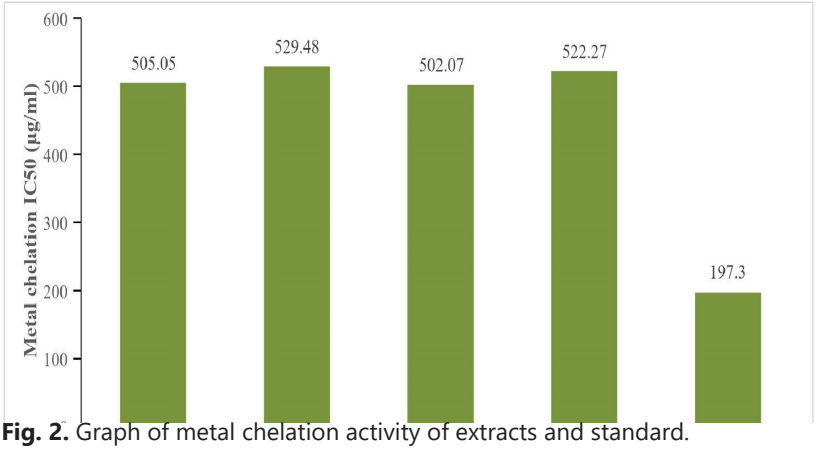

Fig. 2. Graph of metal chelation activity of extracts and standard.

amide, (-)-spathulenol, hexadecadienoate and oleic acid methyl ester demonstrated high gastrointestinal absorption power. Oleic acid methyl ester, petroselinic acid (methyl ester), lupeol, arachidic alcohol, octacosanol and pentatriacontene were forecasted to not intercross the blood-brain barrier (BBB). Some of the selected compounds mainly interact with two isoenzymes of cytochrome (CYP) family, *i.e.*, CYP1A2 and CYP2C19, suggesting their potency, although possessing least toxicity. The GI absorption and drug like qualities in selective compounds of HUFME, AUFME, HUFHE and AUFHE were presented by bioavailablity radar graph (Fig. 3) and boiled-egg prediction (Fig. 4). In the boiled-egg graph, yellow area can permeate via blood-brain

Table 5
Alpha-glucosidase and alpha-amylase inhibitory activity of methanol and hexane extracts from the fruits of *F. auriculata*.

Process Co-products	Alpha glucosidase IC_{50} Mean ± SD (µg/mL)	Alpha amylase IC_{50} value Mean ± SD (µg/mL)
HUFME	245.02±1.81	255.1±1.02
AUFME	257.54±1.38	240.45±1.26
HUFHE	317.8±1.6	290.73±0.546
AUFHE	291.87±1.49	271.46±0.84
Acarbose	230.4±0.32	230.16±0.032

'*' = Standard, Values are means of three replicates ± Standard deviation.

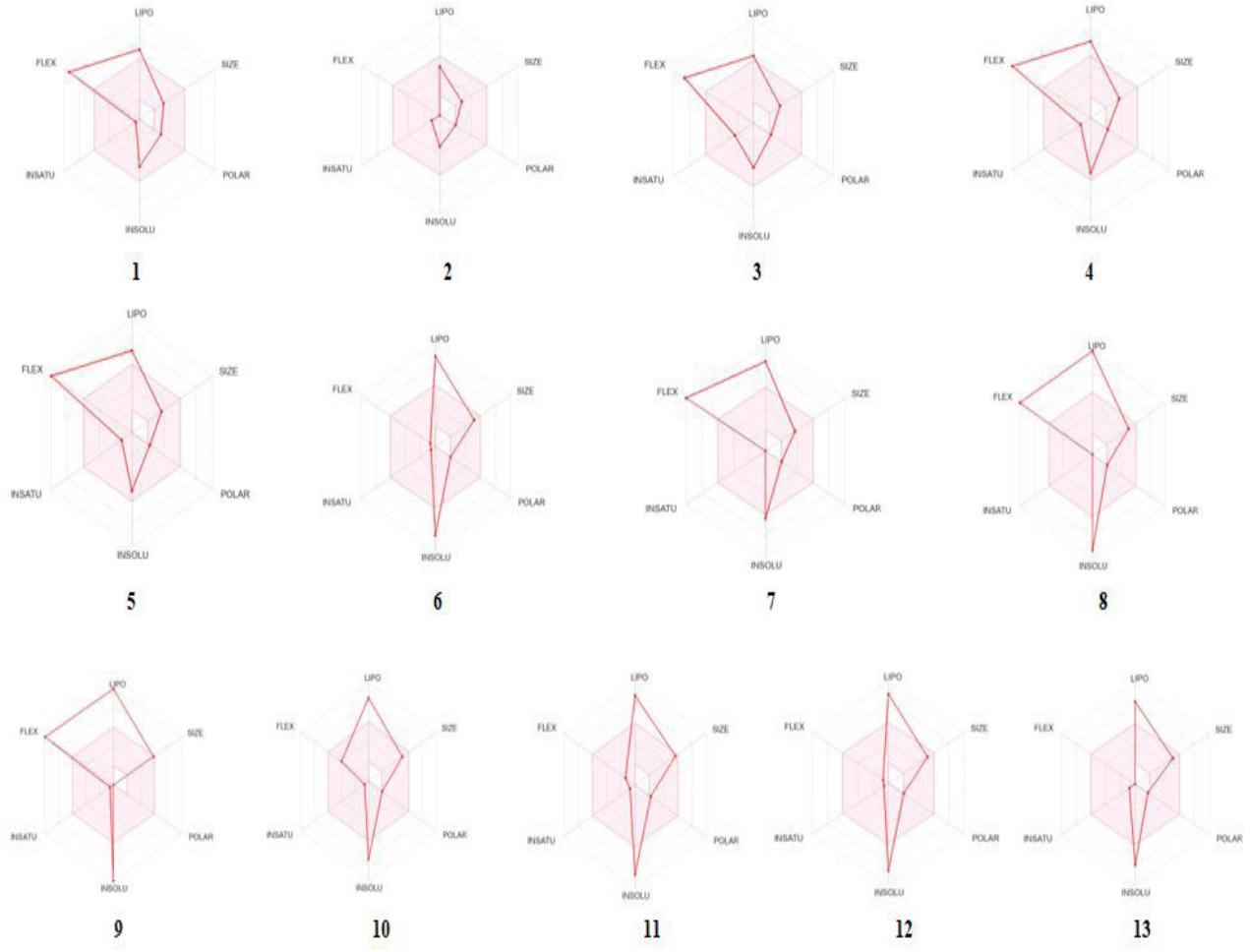

Fig. 3. Bioavailability radar graph of selected compounds (pink area demonstrated the drug likeness action of chosen compounds) 1: palmitic amide, 2: (-)-Spathulenol, 3: Hexadecadienoate, 4: Oleic acid, methyl ester, 5: Petroselinic acid (methyl ester), 6: Lupeol, 7: Arachidic alcohol, 8: Octacosanol, 9: Pentatriacontene, 10: β-Sitosterol, 11: Amyrin, acetate, 12: Triterpene lupeol, 13: β-Amyrone.

Table 6
In silico ADMET prediction of major constituents in AUFME, HUFME, AUFHE and HUFHE.

Entry	1	2	3	4	5	6	7	8	9	10	11	12	13
TPSA * (A2)	43.09	20.23	26.3	26.3	26.3	20.23	20.23	20.23	0	20.23	26.3	20.23	17.07
Consensus * Log Po/w	4.83	3.26	5.05	5.95	5.88	7.26	6.88	9.8	13.34	7.19	7.63	7.26	7.21
Mol wt (g/mol)	255.44	220.35	266.42	296.49	296.49	426.72	298.55	410.76	490.93	414.71	468.75	426.72	424.7
NRB	14	0	13	16	16	1	18	26	32	6	2	1	0
NOHA	1	1	2	2	2	1	1	1	0	1	2	1	1
NOHD	1	1	0	0	0	1	1	1	0	1	0	1	0
WLOGP	4.95	3.39	5.19	6.2	6.2	8.02	7.02	10.14	13.68	8.02	8.74	8.02	8.38
Water solubility	Poorly soluble	Soluble	Poorly soluble	Poorly soluble	Poorly soluble	Insoluble	Poorly soluble	Insoluble	Insoluble	Poorly soluble	Insoluble	Insoluble	Poorly soluble
GI absorption **	High	High	High	High	High	Low	Low	Low	Low	Low	Low	Low	Low
BBB permeant **	Yes	Yes	Yes	No	No	No	No	No	No	No	No	No	No
P-gp substrate **	No	No	No	No	No	No	No	Yes	Yes	No	No	No	No
CYP1A2 inhibitor **	Yes	No	Yes	Yes	Yes	No	Yes	No	No	No	No	No	No
CYP2C19 inhibitor **	No	Yes	No	No	No	No	No	No	No	No	No	No	No
CYP2C9 inhibitor **	No	No	Yes	No	No	No	No	No	No	No	No	No	No
CYP2D6 inhibitor **	No	No	No	No	No	No	No	No	No	No	No	No	No
CYP3A4 inhibitor **	No	No	No	No	No	No	No	No	No	No	No	No	No
Log Kp (cm/s)	-3.23	-5.44	-3.85	-2.82	-3.03	-1.9	-1.53	0.86	4.37	-2.2	-2.25	-1.9	-2.61
Lipinski violation	0	0	1	1	1	1	1	1	1	1	1	1	1
Bioavailability score ***	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55

Drug Likeliness ***, Pharmacokinetics**, Lipophilicity*, TPSA: topological polar surface area, nHA: no. of H-bond acceptor, nHD: no. of H-bond donor, nRB: no. of rotable bond, WLOGP: water partition coefficient, GI: gastrointestinal absorption, BBB: blood-brain barrier, P-gp: permeability glycoprotein, CYP: cytochrome P450, Y-Yes, No-N. Entry: 1: palmitic amide, 2: (-)-Spathulenol, 3: Hexadecadienoate, 4: Oleic acid, methyl ester, 5: Petroselinic acid (methyl ester), 6: Lupeol, 7: Arachidic alcohol, 8: Octacosanol, 9: Pentatriacontene, 10: β-Sitosterol, 11: Amyrin, acetate, 12: Triterpene lupeol, 13: β-Amyrone.

Fig. 4. Boiled-egg graph of selected phytoconstituents.

barrier (BBB) and drug-likeness of selected compounds were shown in bioavailability radar graphs by pink area.A web server ProTox II was used to predict the toxicity parameter of some chosen compounds presented in Table 7. All the selected compounds were predicted to be hepatotoxic and cytotoxic in nature except Palmitic amide. The chosen compounds were predicted not to be carcinogenic, mutagenic and immunotoxic character. The LD_{50} value was also determined to assure the safety of these chosen compounds.

3.6. PCA analysis

PCA is one of the best multivariate statistical tools to interpret the dataset having large number of dimension per observation. In the present work, PCA was used to explore the chemical profiling of compounds in which changes occurred by altitudinal variations, distinct sample can be used in PCA pattern perception. PCA demonstrated the collective contribution rate of variance of PC1 and PC2 could report for 58.1% of variance facts for the change in chemical compounds presented in Fig. 5. The compositional difference in sample was explained in terms of PC1 and PC2 shown in Fig. 6. PC1 contributed 31.7% of variance on positively linked with γ-sitosterol, palmiticamide, hexadecadienoate, α-tocopherol, arachidoic alchol, spathulenol though PC2 makes 26.4% of variance on linked with spathulenol, lupenylacetate, lupeol, pentatriacontene, octacosal and β-sitosterol.
A correlation analysis representation in Fig. 6 shows the phytochemicals and biological activity of *F. auriculata* exhibited a significant correlation exists between them.

4. Concluding remarks

The current research dealt with the GC-MS analysis, phytochemical composition, *in vitro* antioxidant properties, and antidiabetic effects of *F. auriculata* organic extracts. The plant material was collected from Almora and Haldwani in Uttarakhand, India. The GC-MS analysis resulted in identification of 37 bioactive compounds in the unripe hexane fruit extract from Almora region consisting of γ-sitosterol as the major constituent component (15.46%), and 40 bioactive compounds in the unripe hexane fruit extract from Haldwani with γ-sitosterol as the major constituent component (13.4%). Additionally, 24 bioactive compounds were found in the unripe methanol fruit extract from Almora with linoleic acid as the major

Table 7
Toxicity analysis of selected compounds from AUFME, HUFME, AUFHE and HUFHE.

Compounds	Hepatotoxicity		Carcinogenicity		Cytotoxicity		Immunotoxicity		Mutagenicity		Predicted LD_{50} (mg/kg)	Toxicity Class
	Pr	Pb	Pr	Pb	Pr	Pb	Pr	Pb	Pr	Pb		
Palmitic amide	NH	0.82	NC	0.61	NCy	0.99	NI	0.99	NM	0.72	1000	IV
(-)-Spathulenol	H	0.69	NC	0.62	Cy	0.96	NI	0.97	NM	0.93	1190	IV
Hexadecadienoate	H	0.69	NC	0.62	Cy	0.96	NI	0.97	NM	0.93	1190	IV
Oleic acid, methyl ester	H	0.69	NC	0.62	Cy	0.96	NI	0.97	NM	0.93	1190	IV
Petroselinic acid (methyl ester)	H	0.69	NC	0.62	Cy	0.96	NI	0.97	NM	0.93	1190	IV
Lupeol	H	0.69	NC	0.62	Cy	0.96	NI	0.97	NM	0.93	1190	IV
Arachidic alcohol	H	0.69	NC	0.62	Cy	0.96	NI	0.97	NM	0.93	1190	IV
Octacosanol	H	0.69	NC	0.62	Cy	0.96	NI	0.97	NM	0.93	1190	IV
Pentatriacontene	H	0.69	NC	0.62	Cy	0.96	NI	0.97	NM	0.93	1190	IV
?-Sitosterol	H	0.69	NC	0.62	Cy	0.96	NI	0.97	NM	0.93	1190	IV
Amyrin, acetate	H	0.69	NC	0.62	Cy	0.96	NI	0.97	NM	0.93	1190	IV
Triterpene lupeol	H	0.69	NC	0.62	Cy	0.96	NI	0.97	NM	0.93	1190	IV
?-Amyrone	H	0.69	NC	0.62	Cy	0.96	NI	0.97	NM	0.93	1190	IV

Pr: Prediction, Pb: Probability, H: Hepatotoxic, NH: Non-hepatotoxic, C: Carcinogenic, NC: Non-carcinogenic, NCy: Non-cytotoxic, NI: Non-immunotoxic, (Class I: if swallowed, fatal (LD50 ≤ 5), (Class II: if swallowed, fatal (5< LD50 ≤ 50), (Class III: if swallowed, toxic (50< LD50 ≤ 300), (Class IV: if swallowed, harmful (300< LD50 ≤ 2000), (Class V: if swallowed, possibly harmful (2000< LD50 ≤ 5000), (Class VI: non-toxic(LD50 >5000).

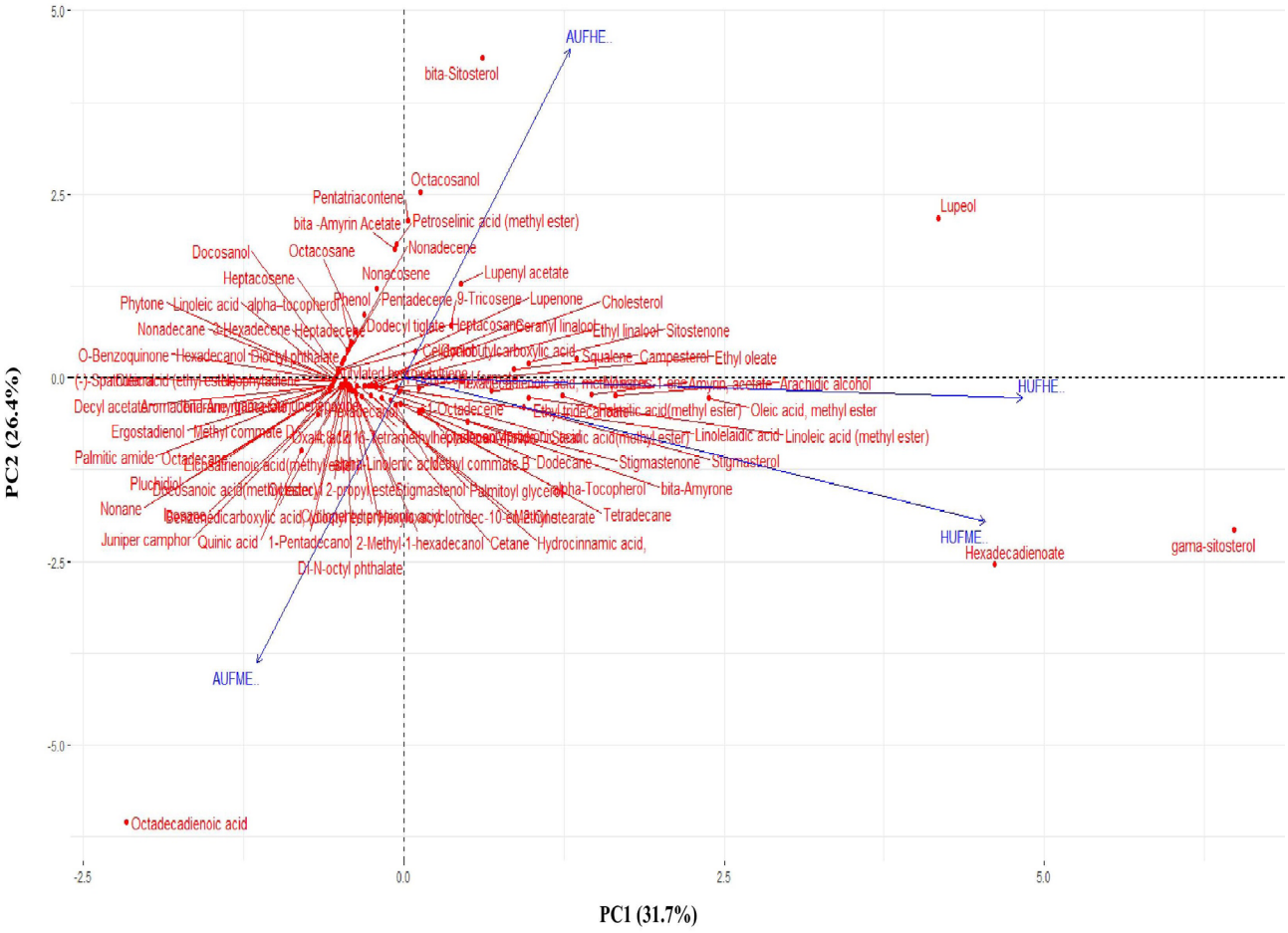

Fig. 5. Principal component analysis (PCA) of phytoconstituents observed in *F. auriculata* extract.

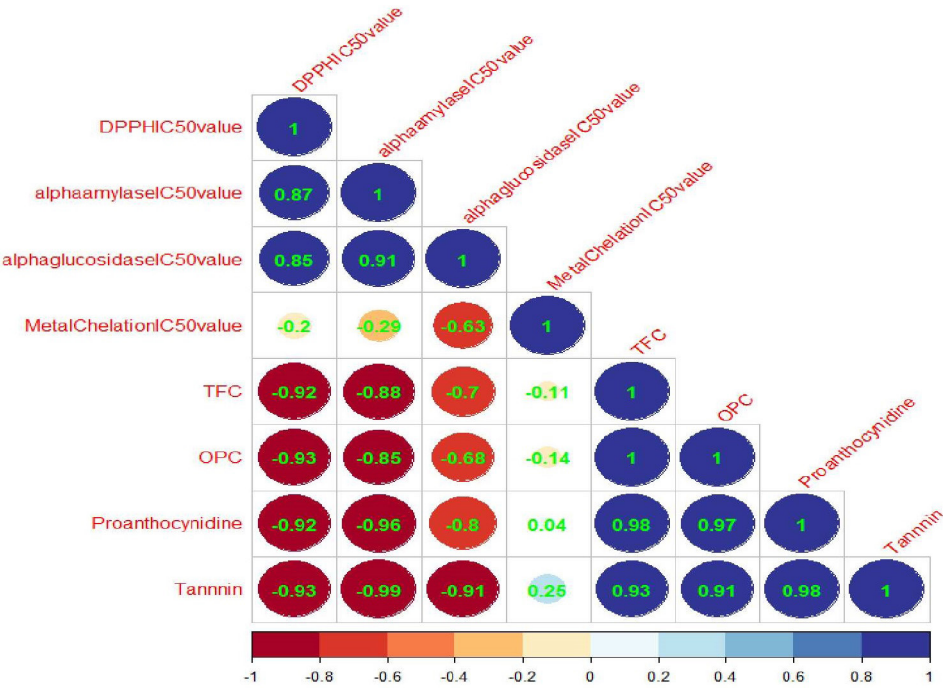

Fig. 6. A correlation study between phytochemicals and biological activity of *F. auriculata* fruit extract.

compound (71.41%), and 23 bioactive compounds were recognized in the unripe methanol fruit extract from Haldwani with hexadecadienoate (26.42%) as the dominant compound. This study revealed that the Haldwani unripe methanol fruit extract exhibited higher total phenolic, flavonoid, and tannin content, whereas the Almora unripe methanol fruit extract showed an IC_{50} of 447.45 ± 0.53 µg/mL when using DPPH radical scavenging activity assay. The Haldwani unripe hexane fruit extract displayed significant metal chelation activity (IC_{50} = 502.07 ± 2.50 µg/mL), as well. According to our findings, both Almora and Haldwani unripe methanol extracts of *F. auriculata* fruit could serve as potent antidiabetic effects. Furthermore, an ADMET study suggested that *F. auriculata* could be a valuable source of bioactive compounds with various biological benefits. The presence of polyphenols, flavonoids, and fatty acids in the fruit could also verify the relevant pharmacological properties.

It is concluded that Almora unripe fruit methanol extract has prominent biological and pharmacological potential than Haldwani unripe fruits. The fruits can be used to cure and reduce oxidative stress induced by free radicals. The antidiabetic potential focuses on the inhibitory action on α-amylase and α-glucosidase. Present study reported a potential mechanism of *F. auriculata* fruit and recommend that outcome of the fruit is due to the inhibition of digestive enzymes. However, the presence of phenols and flavonoids signifies the multiple biological efficacies of this plant. Some new bioactive compounds like quinic acid and other unsaturated fatty acids have been found, which will be researched deeply in the future along with their pharmacological activity. Natural antioxidants are extensively found in food, medicinal plants and herbs. Such natural antioxidants, commonly polyphenols, flavonoids and various other phytoconstituents, exhibits a endless biological significance and pharmacological properties and are also promising therapeutic agents, including anti-inflammatory, antidiabetic, antimicrobial and herbicidal activity. In the future, effective drugs from plant-derived components could be isolated and formulated for human ailments and cures.

Author Contribution statement

Conceptulization and design of study was done by Shishir Tandon and Viveka Nand. Literature search was performed by Shishir Tandon and Garima Tamta. The first draft of the manuscript was prepared by Garima Tamta and Nisha Mehra. *In silico* study was done by Nisha Mehra. Statistical analysis was performed by Manish Pant. Viveka Nand and Vinita Gouri gave suggestion to finalize the manuscript. All authors read and approved the final manuscript.

Data availability

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Competing interests

The authors declare that there is no competing interests.

Conflict of interest

The authors declare that there is no conflict of interest.

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