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Original article

# Analysis of medicinally important phytocompounds from *Argemone mexicana*

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ABSTRACT

The present study was aimed to evaluate the total phenolic contents (TPC), total flavonoid contents (TFC) and GC-MS analysis for the presence of some medicinally important compound from *Argemone mexicana* leaf, stem and flower extracts. Plant extracts of *Argemone mexicana* leaf, stem and flowers were prepared using Soxhlet extraction. Preliminary detection of phytocompounds was carried out. Total phenolic content of plant extracts was analysed using Folin-ciocalteu reagent and total flavonoid content using aluminium chloride. Gas Chromatography Mass Spectroscopy was (GC-MS) was performed to identify phytocompounds present in plant extracts using National Institute of Standards and Technology (NIST) library. The highest total phenolic content was reported in *Argemone mexicana* stem extract and highest flavonoid content was reported in flower extract. A wide range of fatty acids and phytocompounds were identified which are utilized in antibacterial, antifungal, anti-inflammatory and antimycotic activities. The study concludes that *Argemone mexicana* have many biologically important compounds, so it can be recommended as a plant of pharmaceutical importance.


## 1. Introduction

*Argemone mexicana* is a flowering plant belong to Papaveraceae family. *Argemone mexicana* L. (Papaveraceae) is commonly known as Mexican Poppy and Premathandu in Tamil. The plant grows between 0.3 and 1.2 meters (Hakim, 1954; Schwarzbach and Kadereit, 1999). It is a small herbaceous plant with latex. The plant is abundant in Mexico and now has been found in India, the United States, Bangladesh and Ethiopia. It occurs in agricultural and wasteland weed areas in almost every part of India and Bangladesh (Das and Misra,1987; Mukherjee and Namahata, 1990).

Medicinal plants are being utilized as effective source of medicines as modern or traditional medicines. Some antibiotics cause development of multi drug resistance capacity of pathogens. The herbal medicines can be used as a possible way to treat diseases caused by multi drug resistant bacteria. Thus, traditional medicines are safe treatment of microbial infections, show no side effects and are biodegradable. According to WHO, about 80% people in the World's use plants for their primary health care (Khan and Bhadauria, 2017a).

*Argemone mexicana* plant parts have been used in Ayruveda and Yunani as purgative and sedative and for treatment of skin diseases (Chandhurirai et al., 1985). The plant has some important biological activities such as antimicrobial, larvicidal. chemosterilant, nematicidal and wound healing capacity (Shaukat et al., 2002). In Mexico, the seeds are used as a remedy to snake poisoning (Bhattacharjee et al., 2006). In India, the smoke of the seeds is used to relieve toothache. The tincture of the plant is encountered in oral treatment of bronchitis and whooping cough in Homeopathy (Kala, 2005).

*Argemone mexicana* plant parts have been used to treat many infectious diseases. The leaves and stems (aerial part) of the plants are used as a remedy in malaria, dropsy and icterus and also used for analgesic, antispasmodic, anti-parasitic and narcotic effects (Singh et al., 2009; Graz et al., 2010). The fresh juice of *A. mexicana* leaves and the latex is reported to be used as a disinfectant when applied externally on open wounds and cuts. The fresh milky seed extract is used in cure of leprosy, warts, cold sores diuretic, jaundice, malarial fever, wound healing, skin diseases, scorpion sting, itches, anti-inflammatory and various anti-poison activities. These activities are due to presence of secondary metabolites and some protein-dissolving substances (Alagesaboopathi, 2009; Panghal

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et al., 2010). The secondary metabolites responsible for medicinal activity of *A. mexicana* include polyphenols, flavonoids, alkaloids, phenols tannins and saponins (Ji et al., 2011).

Thus, the present research was attempted to investigate phytochemical analysis of *Argemone mexicana* leaf, stem and flower extracts using GC-MS analysis and analysis of Total Phenolic and Total Flavonoid Content using UV-VIS spectrophotometer.

## 2. Materials and methods

### 2.1. Materials

The *Argemone mexicana* plants were collected from Sitapura area of Jaipur city, Rajasthan, India. The collected plant was identified and confirmed by herbarium, department of botany, University of Rajasthan, Jaipur. Methanol, petroleum ether, Diethyl ether, ethyl acetate and separating funnel were purchased from Himedia Labs, Jaipur under Laboratory Grade (LR).

### 2.2. Experimental work

#### 2.2.1. Preparation of plant material

The collected plants were washed successively with tap water to eliminate the soil and dust. Then leaf, roots and flowers were separated from plants and shade dried for 10 days. Then dried plant parts were grounded separately to make powder and stored in air tight containers separately.

#### 2.2.2. Preparation of samples

The powdered samples of *Argemone mexicana* leaf, stem and flowers were extracted in Soxhlet extraction unit (Subramanian and Nagarajan, 1969). Extraction was done in water and 80% methanol for 24 h on heating mental. The plant extracts were separately filtered by Whatman filter paper No.1.

The filtrate was again separated into ether layer and below side water layer using a separating funnel by adding 7% $H_2SO_4$ and ether. These layers were filtered and separated ether layers were evaporated at room temperature (Bhadauria and Kumar, 2011). The obtained extracts were stored and analysed by GC-MS.

#### 2.2.3. Preliminary phytochemical screening

The methanol extracts of *A. mexicana* leaf, stem and flowers were subjected to preliminary phytochemical using standard procedures as described by Ugochukwu et al. (2013). The preliminary phytochemicals were analyzed in order to detect presence or absence of some important phyto-compounds.

*2.2.3.1. Test for flavonoids.* The extract was treated with 1 ml of sodium hydroxide; appearance of yellow colour indicated the presence of flavonoids (Harborne, 1973).

*2.2.3.2. Test for phenolic compounds.* 1% of ferric chloride was added with 5 ml of plant extract. A blue-green colour indicated the presence of phenolic compounds.

*2.2.3.3. Test for terpenoids and steroids.* Plant extract was taken in a test tube with 0.5 ml of chloroform. The concentrated sulphuric acid solution was then added slowly. The red to violet colour indicated for terpenoid presence and green bluish colour indicated steroids presence in plant (Siddiqui and Ali, 1997).

*2.2.3.4. Test for proteins and Amino acids.* In this test 1 ml of sodium hydroxide solution (40%) was assorted with 2 drops of copper sulphate (1%). When the solution becomes blue in color, 1 ml of the plant extract was added. Formation of purple color showed the presence of proteins.

*2.2.3.5. Test for saponins.* To, the plant extract, 10 ml of distilled water was added and shaken vigorously for 2 min. Appearance of a foam layer indicated presence of saponins.

*2.2.3.6. Test for reducing sugars.* In this test, 5 ml of Fehling's solution I and II (1:1 ratio) was mixed with 2 ml of plant extract. The mixture was boiled for 5 min. A red precipitate demonstrated the presence/absence of reducing sugars (Brain and Turner, 1975).

*2.2.3.7. Test for glycosides.* In a test tube, 5 mL of each extract, 2 mL of glacial acetic acid and one drop of ferric chloride solution (1%) were added and mixed thoroughly. Finally, 1 mL of 1 N $H_2SO_4$ was added. A brown ring at the interface demonstrated the presence of glycosides.

*2.2.3.8. Test for resins.* 10 mL of the plant extract was added to equal volume of 1% copper acetate solution in a test-tube. The mixture was agitated vigorously. A separate green color exhibited the presence/absence of resins (Harborne, 1998).

#### 2.2.4. Analysis of total phenol content

Total phenol concentration was determined using Folin-Ciocalteu reagent, as described by McDonald et al. (2001) with little modifications. An aliquot of 0.1 ml crude extract and 0.9 ml methanol was taken in a test tube with 10-fold diluted Folin-Ciocalteu reagent. Then 3 ml of 10% sodium carbonate was added to mixture. The mixture was held at room temperature for 90 min and then absorbance was taken at 765 nm using UV-VIS spectrophotometer. The total phenolic content measurements were taken in triplicates. Gallic acid was employed as the standard phenol. The total phenolic content was calculated by standard curve of Gallic acid as standard prepared with methanol in the range 20–200 μg/ml (R2 = 0.987). Experimental results are expressed as means ± standard deviation (SD). Total phenol content was exhibited in terms of Gallic acid equivalent (mg GAE /g) of plant material.

#### 2.2.5. Analysis of total flavonoid content

Total flavonoid content was determined by using previously used protocol of aluminium chloride colorimetric technique (AlCl3) according to the known method given by Chang et al. (2002) with slight modifications. 1 ml of plant extract was brought in a test tube and mixed with 100 μl of 1 M aluminum chloride solution and 100 μl potassium acetate. 2.8 ml methanol was added in test tube for a final volume of 4 ml. The reaction was sustained at room temperature for 30 min for appearance of stable Yellow color in reaction mixture. Quercetin was used as the standard flavonoid. The absorbance was taken at 510 nm by UV-VIS spectrophotometer. Total flavonoid content readings were taken in triplicates. Total flavonoid content was calculated by the standard curve of Quercetin prepared with methanol in range 0.5–5.0 mg/ml (R2 = 0.991) and was shown in milligram of Quercetin equivalents/gdw (mg QE/g) plant extract. Experimental results were expressed as means ± standard deviation (SD) respectively.

#### 2.2.6. Interpretation of mass spectrum

The plant extracts were subjected to GC-MS analysis. For this 1 mg/ml concentration of the extracted samples was prepared in methanol. The sample was kept in sterilized glass vial and analyzed for GC-Mass Spectroscopy.

*2.2.6.1. GC-MS condition.* GC-MS was accessed using SHIMADZU QP2014 ULTRA apparatus operated in EI mode at 70ev. A Restek-5MS column (30 m × 0.25 mm × 0.25 μm) was used. The oven

temperature was projected at 60° raised to 280 °C at 5 °C min-1 and held for 2 min, then 250 °C to 280 °C and held for 14 min. The injector temperature was 290 °C with normal injection mode. The flow rate of carrier gas, helium was 1.00 ml min-1. Total running time for GC was 30 min. The compounds were identified by comparing the mass spectral data with data stored in GC-MS library (Khan and Bhadauria, 2017b).

## 3. Results

Assessment of the various phytochemicals in *Argemone mexicana* leaf, stem and flower extract was done on the methanolic extracts using standard procedures. Preliminary detection of phytochemicals in the plant crude extracts was done by applying different tests for saponins, phenols, flavonoids, terpenoids, steroids, proteins, aldehyde, ketones, reducing sugar, glycosides, and resins by standard phytochemical tests. The plant showed both presence and absence of various compounds (Table 1). In the preliminary detection of phytochemical present in plant extract of *A. mexicana* leaf, stem and flower were found positive for the phytochemical constituents namely saponins, phenols, flavonoids, terpenoids, steroids, proteins, aldehyde and ketones and found negative for reducing sugar, glycosides, and resins.Table 2.

The highest total phenolic content was reported in stems (28.5 ± 1.15 mg GAE/g of plant extract) followed by flower (22.23 ± 0.61 mg GAE /g) and leaves (20.89 ± 0.89 mg GAE /g) of *Argemone mexicana*. (Table 2)

The highest flavonoid content in *Argemone mexicana,* was estimated in flowers (41.76 ± 0.74 mg QE/g of plant extract) followed by leaf (30.59 ± 1.27 mg QE/g) and stem (22.83 ± 0.83 mg QE/g). The data of total phenolic content and total flavonoid content *of A. mexicana* leaf, stem and flower is given in Fig. 1. The maximum total phenolics /total flavonoids ratio was recorded in stems (1.24), followed by leaves (0.68) and flower (0.53). The phenolics /total flavonoids ratio indicate that flavonoids may be primarily responsible for biological activities of *A. mexicana.*

### 3.1. Identification of components by GC-MS

Identification of phytocompounds was based on the principles of molecular weight (MW), retention time (RT), molecular formula (MF) and concentration (peak area%). It was done in order to determine some compounds present in plants having any medicinal value. The Gas chromatography mass spectrum of the sample was interpreted using the database of National Institute Standard and Technology (NIST) having more than 2,00,000 patterns. The database was read using GC-SOLUTION software. For identification of any unknown compound, its spectrum is compared to spectrums database stored in NIST-11 library for similarity (Figs. 2, 3, 4). Each compound name was confirmed with Similarity Index (SI). Similarity Index is the similarity percentage of any compound to GC-MS library. The compound showing highest Similarity Index was confirmed as final compound. The names of compounds were also ascertained with molecular weight of the isolated compounds (Kumaravel et al., 2010).

**Table 2**
Total phenolic content and total flavonoid content in *Argemome mexicana* plant parts.

| Plant Part | Total Phenolic content (mg GAE/ gdw)[a] | Total flavonoid content (mg QE/ gdw)[b] | Total phenolics/ Total flavonoids |
|---|---|---|---|
| Leaves | 20.89 ± 0.89 | 30.59 ± 1.27 | 0.68 |
| Stems | 28.5 ± 1.15 | 22.83 ± 0.83 | 1.24 |
| Flower | 22.23 ± 0.61 | 41.76 ± 0.74 | 0.53 |

a = mg Gallic acid equivalent/gm of plant extract); b = mg quercetin equivalent/gm of plant extract.
Each value is expressed as mean ± S.E (Standard Error) (n = 3).

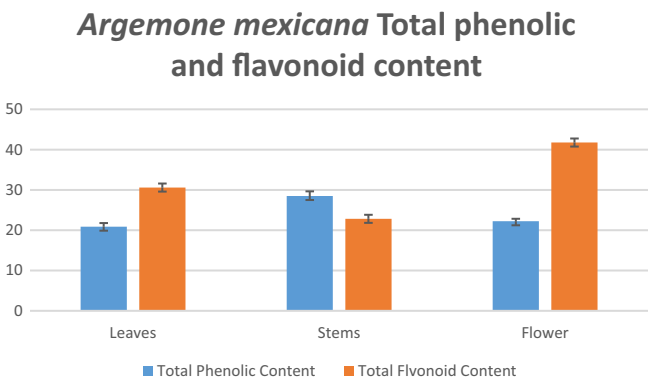


**Fig. 1.** *Argemone mexicana* Total phenolic and flavonoid content.

#### 3.1.1. Compounds identified in A. Mexicana leaf extract

Fig. 2 shown in the GC/MS analysis of leaf extracts of *A. mexicana* revealed the existence of several compounds. The compounds identified in *Argemone mexicana* leaf extracts are 9,12-Octadecadienoic acid (Z,Z)- (32.33%), n–Hexadecanoic acid, methyl ester (24.40%) 9-Octadecenoic acid, methyl ester, (E)- (17.41%), 9,12,15-Octadecatrienoic acid (9.20%), Stearic acid, methyl ester (5.57%), Tetradecanoic acid, methyl ester (2.31%), 9-Eicosene, (E)- (2.06%), Bis(2-ethylhexyl) phthalate (1.41%) and 3-Octadecene, (E)-(1.15%) (Table 3).

#### 3.1.2. Compounds identified in A. Mexicana stem extract

Fig. 3 shows the presence of various compounds in *A. mexicana* stems extract. The major compounds reported in *Argemone mexicana* stems are Hexadecanoic acid, methyl ester (21.45%), 9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- (15.25%), 9,12-Octadecadienoic acid (Z,Z)- (12.10%), 3-Octadecene, (E)-

**Table 1**
Preliminary phytochemical screening of *Argemone mexicana.*

| Sr. No. | Test | Leaf | Stem | flower |
|---|---|---|---|---|
| 1 | Flavonoid test | +ve | +ve | +ve |
| 2 | Phenolic test | +ve | +ve | +ve |
| 3 | Reducing sugars test | −ve | −ve | −ve |
| 4 | Aldehyde & ketone test | +ve | +ve | −ve |
| 5 | Glycosides test | −ve | −ve | −ve |
| 6 | Terpenoids test | +ve | +ve | +ve |
| 7 | Protein and Amino acids test | +ve | +ve | +ve |
| 8 | Saponins test | +ve | +ve | −ve |
| 9 | Resins test | −ve | −ve | −ve |

+ve:Positive, −ve: Negative.

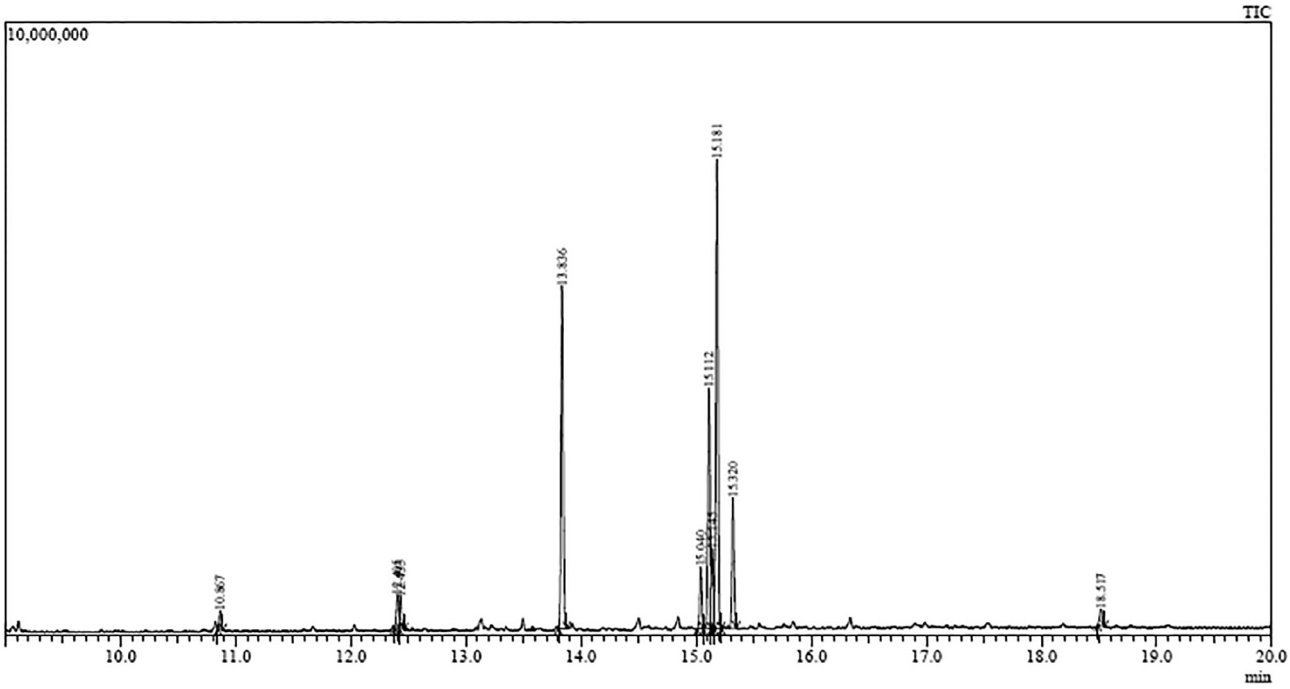


**Fig. 2.** GC–MS chromatogram of *Argemone mexicana* leaf extract.

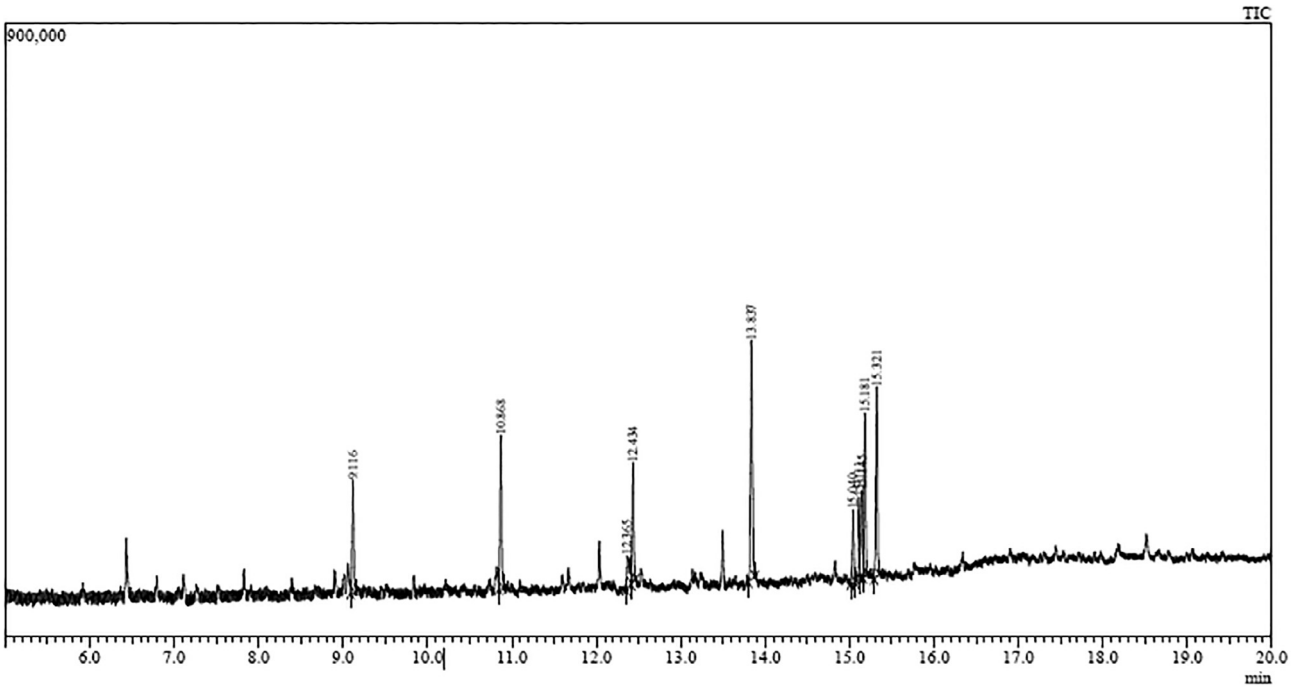


**Fig. 3.** GC–MS chromatogram of *Argemone mexicana* stem extract.

(11.25%), 3-Hexadecene, (Z)- (8.52%), 1-Heptadecene (8.43%), 9-Octadecenoic acid, methyl ester, (E)-(6.96%), Heptadecanoic acid, 10-methyl-, methyl ester (5.92%) and 2-n-dodecylphenol, trifluoroacetate ester (4.78%) (Table 4).

### 3.1.3. Compounds identified in A. Mexicana flower extract

Similarly, *A. mexicana* flower extract revealed the existence of several compounds (Fig. 4). The major compounds identified in *Argemone mexicana* flower extract are 9,12,15-Octadecatrienoic acid, (Z,Z,Z)- (26.87%), n–Hexadecanoic acid, methyl ester (22.77%), 9,12-Octadecadienoic acid (Z,Z)- (13.56%), Oleic Acid (10.16%), Palmitic acid, methyl ester (5.41%), n-Tetradecanoic acid (5.23%), 9,12,15-Octadecatrienoic acid, methyl ester (4.28%), Linoleic acid, methyl ester (4.24%), 2-hydroxy-1-(hydroxymethyl) ethyl ester (1.83%), 3-(4-hydroxyphenyl)-2-Propenoic acid, (1.55%) and 1,4-Benzenediol (1.37%) (Table 5).

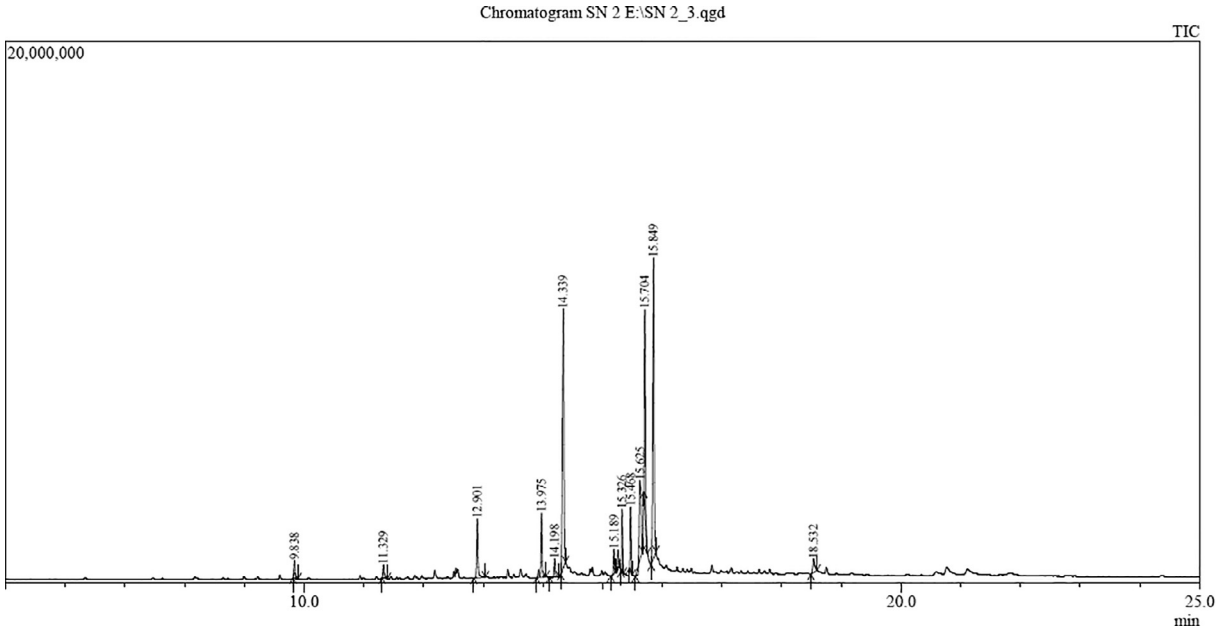


**Fig. 4.** GC–MS chromatogram of *Argemone mexicana* flower extract.

**Table 3**
Phytocomponents identified in *Argemone mexicana* leaf extract.

| Peak No. | R. Time | Area | Area% | Name of compound | Similarity Index (SI) | Molecular Weight |
|---|---|---|---|---|---|---|
| 1 | 10.867 | 346,746 | 1.15 | 3-Octadecene, (E)- | 97 | 252 |
| 2 | 12.405 | 695,022 | 2.31 | Tetradecanoic acid, methyl ester | 91 | 242 |
| 3 | 12.435 | 619,053 | 2.06 | 9-Eicosene, (E)- | 96 | 280 |
| 4 | 13.836 | 7,348,312 | 24.40 | n-Hexadecanoic acid, methyl ester | 96 | 270 |
| 5 | 15.112 | 5,241,925 | 17.41 | 9-Octadecenoic acid, methyl ester, (E)- | 95 | 296 |
| 6 | 15.145 | 1,677,415 | 5.57 | Stearic acid, methyl ester | 89 | 298 |
| 7 | 15.181 | 9,735,572 | 32.33 | 9,12-Octadecadienoic acid (Z,Z)- | 93 | 294 |
| 8 | 15.320 | 2,769,143 | 9.20 | 9,12,15-Octadecatrienoic acid | 95 | 292 |

**Table 4**
Phytocomponents identified in *Argemone mexicana* stems extract.

| Peak No. | R. Time | Area | Area% | Name of compound | Similarity Index (SI) | Molecular Weight |
|---|---|---|---|---|---|---|
| 1 | 9.116 | 219,360 | 8.52 | 3-Hexadecene, (Z)- | 97 | 224 |
| 2 | 10.868 | 289,837 | 11.25 | 3-Octadecene, (E)- | 97 | 252 |
| 3 | 12.365 | 123,246 | 4.78 | 2-n-dodecylphenol, trifluoroacetate ester | 55 | 358 |
| 4 | 12.434 | 217,279 | 8.43 | 1-Heptadecene | 96 | 238 |
| 5 | 13.837 | 552,544 | 21.45 | n-Hexadecanoic acid, methyl ester | 95 | 270 |
| 6 | 15.111 | 179,204 | 6.96 | 9-Octadecenoic acid, methyl ester, (E)- | 93 | 296 |
| 7 | 15.145 | 152,537 | 5.92 | Heptadecanoic acid, 10-methyl-, methyl ester | 90 | 298 |
| 8 | 15.181 | 311,697 | 12.10 | 9,12-Octadecadienoic acid (Z,Z)-, methyl ester | 91 | 294 |
| 9 | 15.321 | 392,946 | 15.25 | 9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- | 95 | 292 |

**Table 5**
Phytocomponents identified in *Argemone mexicana* flower extract.

| Peak No. | R. Time | Area | Area% | Name of compound | Similarity Index (SI) | Molecular Weight |
|---|---|---|---|---|---|---|
| 1 | 9.838 | 906,733 | 1.37 | 1,4-Benzenediol | 97 | 110 |
| 2 | 11.329 | 639,276 | 0.97 | Dodecanoic acid | 96 | 200 |
| 3 | 12.901 | 3,456,177 | 5.23 | n-Tetradecanoic acid | 96 | 228 |
| 4 | 13.975 | 3,577,193 | 5.41 | Palmitic acid, methyl ester | 96 | 270 |
| 5 | 14.198 | 1,021,124 | 1.55 | 3-(4-hydroxyphenyl)-2-Propenoic acid | 96 | 178 |
| 6 | 14.339 | 1,548,353 | 22.77 | n-Hexadecanoic acid | 97 | 270 |
| 7 | 15.326 | 2,802,110 | 4.24 | Linoleic acid, methyl ester | 95 | 294 |
| 8 | 15.468 | 2,828,532 | 4.28 | 9,12,15-Octadecatrienoic acid, methyl ester | 95 | 292 |
| 9 | 15.625 | 6,712,328 | 10.16 | Oleic Acid | 95 | 282 |
| 10 | 15.704 | 8,961,769 | 13.56 | 9,12-Octadecadienoic acid (Z,Z)- | 96 | 294 |
| 11 | 15.849 | 17,754,995 | 26.87 | 9,12,15-Octadecatrienoic acid, (Z,Z,Z)- | 97 | 278 |
| 12 | 18.532 | 1,207,449 | 1.83 | 2-hydroxy-1-(hydroxymethyl) ethyl ester | 92 | 330 |

## 4. Discussion

The preliminary phytochemical screening is the simplest method for detection of secondary metabolites in plant extract. The methanolic and ethanolic extract of *A. mexicana* leaves contains phenols, flavonoids, proteins, tannins, sterols/terpenes and alkaloids (Ibrahim and Ibrahim, 2009). In the preliminary phytochemical screening *A. mexicana* leaf, stem and flower methanol extracts showed positive results for most of the medicinally important phytochemical constituents attributed for curative, antibacterial and antifungal activity (Hussain et al., 2011). Similarly, Gali et al. (2011) related the anticancer effects of flavonoids to methanol extract of *A. mexicana* L. leaves to validates the traditional use of the plant against Cancer. The preliminary detection of similar types of phytocompounds were also reported by Jaliwala et al. (2011).

The results of present study indicated considerable amount of total phenolic content and total flavonoid content. The highest Total phenolic content was recorded in stems and highest total flavonoid content was recorded in flower extract. Methanol is found to be the best solvent to exhibit high phenolic content in *A. mexicana* (Apu et al., 2012). According to Verma et al. (2010) flavonoids and alkaloids seem to be most likely compounds eliciting *in vitro* cytotoxicity effect. The phenolic compounds are reported to show as scavengers of Reactive Oxygen Species (ROS), antioxidant and anti-inflammatory activities (Sivanandham, 2011). The flavonoids are also medicinally important and many researchers have reported flavonoid compounds to exhibit analgesic, anti-inflammatory, antioxidant, anti-arthritic and immunomodulatory properties (Gill et al., 2011).

In this study methanol extract of *Argemone mexicana* leaves, stems and flowers were analyzed for the presence of active bioactive compounds by GC-MS analysis with their spectrum, retention time, molecular weight and similarity index. The mass spectrum of each compounds was compared with NIST-11 data base and gave more than 90% match resulting in confirmatory compound match. The major compounds reported in GC-MS analysis of *A. mexicana* leaf, stem and flower were fatty acids and heterocyclic compounds (Gunstone et al., 1977). Oleic acids, Tetradecanoic acid, Pentadecanoic acid, Hexadecanoic acid and Octadecanoic acid are some important known fatty acids which are acknowledged for antibacterial and antifungal activity (McGraw et al., 2002; Seidel and Taylor, 2004). The compound Hexadecanoic acid, Dodecanone, 9-Octadecenoic acid, 9,12-Octadecadienoic acid, Stearic acid, Tetradecanoic acid, Oleic acid and 3-Octadecene were found as major compounds in *A. mexicana* plant extract and similar compounds were also reported by Kiran et al. (2017); Ponnusamy et al. (2018). Besides medicinal properties 9,12-Octadecadienoic acid (Linoleic acid), acid, Octadecenoic acid and Hexadecanoic acid are also used as omega-6 fatty acid, emulsifying agent and cosmetic ingredients respectively (Powder-George and Mohammed, 2018). The activity of compound was identified from Dr. Duke's Phytochemical and Ethnobotanical database (Duke and Beckstrom-Sternberg, 1994).

## 5. Conclusion

*Argemone mexicana* plant and its parts are utilized for cure of asthma, malaria, dropsy, bronchitis, bio-insecticides and skin diseases. There are limited reports available on the extraction and identification of biological important compounds from *Argemone mexicana*. Some important phytocompounds were screened preliminarily by standard phytochemical tests. In this study, methanol extract of *Argemone mexicana* leaf, stem and flowers were quantitatively analyzed for total phenolic and flavonoid content and then active bioactive compounds of plants were evaluated by GC-MS analysis. The major compound identified by GC-MS belong to fatty acids. These identified phytocompounds are assumed to be responsible for eliciting the traditional medicinal activity of *A. mexicana*. The present study is significant because there is less literature available on *Argemone mexicana* phytochemical analysis.

## Conflict of interests

We declare that there is no conflict of interest.

## Acknowledgment

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