REPORT

Antioxidant, cytotoxicity, protein kinase inhibition and antibacterial activities of *Fragaria × ananassa* leaves

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Abstract: *Fragaria × ananassa* leaves extracts prepared in different solvents were subject for antioxidative, cytotoxicity, protein kinase inhibition and antibacterial activities. The extracts showed varying activities depending upon solvent used for extraction. Combined effect of methanol and ethyl acetate showed maximum antioxidant and reducing power potential (207.65±6µg AAE/mg and 88.58±20µg AAE/mg, respectively). Maximum DPPH (2,2-diphenyl-1-picryl hydrazyl) free radical scavenging activity was calculated by when methanol: chloroform and acetate fractions were used (87.68% and 86.88% inhibition, respectively). Total phenolics varied from 186 to 1.91µg AAE/mg while total flavonoids also significantly varied among the extracts. The extracts also showed significant activities against brine shrimp larvae and bacterial strains tested. The study concludes that *Fragaria × ananassa* leaves can be a good source for isolation of active phytochemicals to be used in different industries.

Keywords: *Fragaria × ananassa* leaves extracts, antioxidant, DPPH, antibacterial, protein kinase, cytotoxicity

INTRODUCTION

Plants have the ability to carry out a vital natural function by using an extensive diversity of chemical compounds, and to protect against attack from predators. Most of the chemicals which are synthesized by plants as secondary metabolites are useful to maintain the health of mankind and other animals as well. Secondary metabolites have their own specific importance and these include alkaloid, flavonoids, terpenoids and such other compounds, which performed different functions (Lai and Roy, 2004; Tapsell, 2006). Many of these secondary metabolites or phytochemicals have valuable effects when consumed by humans and their usage are effective in the treatment of different diseases. Approximately 12,000 such compounds have been isolated so for (Sahreen *et al*., 2011).

Strawberry (*Fragaria × ananassa*) is one of the dominant medicinally important plant widely grown in the world belonging to the family of Rosaceae. Its fruit is highly appreciated for its characteristic aroma, bright red color, juicy texture, and sweetness. Strawberry and other fruits and vegetables are worthy source of natural antioxidants particularly the existence of extremely phenolic compounds known as polyphenolic, which potentially protect the human body from diverse disease and infections like damage and delay senescence caused by oxidative stress (Seeram *et al*., 2006). As compared to other fruit plants of the Rosaceae family including apple, peaches, plums, raspberries and pears, strawberry plant has particularly rich secondary metabolite composition and consist of hundreds of non-volatile and volatile compounds (Hanhineva *et al*., 2011). Previous study of strawberry plant shown to possess high *in vitro* antioxidant activity that have been positively interconnected with highly phenolic compounds and, specifically, anthocyanin's polyphenolic, quantitatively most important in the strawberry plant (Wang and Jiao, 2000; Wang and Lin, 2000).

Since recent studies have shown that a diet rich in antioxidant reduces risk of cancer and of cardiovascular diseases, strawberries can be considered as an important dietary source because of their high level of vitamin C, anthocyanins, ellagic acid and other antioxidant compounds (Koponen *et al*., 2007; Jakobek *et al*., 2007; Koehler *et al*., 2015).

This research work was conducted to find the phytochemical composition, cytotoxic potential, and antioxidant capacity of *F. ananassa* leaves. Phytochemical screening of various fractions was made and different kinds of assays were screened out e.g. antimicrobial, antioxidant, cytotoxic, phenolics, flavonoids and protein kinas inhibition assay. Beside the results from present study would also provide a better understanding on the antioxidant activity of *F.× ananassa* so that it would be identified for further investigations and research and

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therefore, developed into value-added foods and nutraceuticals for the assistance of mankind.

MATERIALS AND METHODS

Plant material and extract preparation

The leaves of the *Fragaria x ananassa* plant were collected from District Mardan Khyber Pakhtoon Khaw, Pakistan in June 2014 and the plant was identified by its local name and later identified by Dr. Rizwana Alim Qureshi (Taxonomist, Quaid-i-Azam University Islamabad) and submitted in the Herbarium at Quaid-i-Azam University Islamabad (voucher specimen number PHM-495). The leaves (4.0kg) were collected and dried under shade condition to obtain 1.0kg dry sample. The dried sample was powdered with the help of a grinder and prepare for the next step of solvent extraction using cold maceration technique. For extraction different solvent were used like methanol, ethanol, chloroform n- hexane, ethyl acetate, water and acetone; alone or in combination 1:1. Extracts were prepared by soaking 100g of the dry powdered plant materials in 1l of each solvent at room temperature for 48 h. The extracts were filtered after 48 h through cotton cloth and then through Whatmann filter paper 1. Then rotary evaporator was used for concentration of the extracts. The percentage yield of the extracts was ranged from 9-21% w/w.

Evaluation of antioxidant activity

Phosphomolybdenum based total antioxidant capacity determination, DPPH-radical scavenging activity and reducing power assay was tested to evaluate antioxidant potential according to the method described below.

Phosphomolybdenum based total antioxidant capacity (TAC) determination

Total antioxidant activity of *F. × ananassa* plant extract was evaluated by incubating 100µl of 4mg/ml DMSO stock solution of each extract with reagent solution, including of 4mM ammonium moybdate, 28mM sodium phosphate and 0.6M sulfuric acid, at 90°C for 90min. Absorbance of each extract was measured using UV/VIS-DAD spectrophotometer at 645 nm. Ascorbic acid is used as standard (positive control) and the reagent without sample is used as negative control. All the experiments were repeated three times (Prieto *et al.*, 1999).

Total reducing power assay (TRP)

The measurement of reducing power capability of extract was estimated by following the method of Oyaizo *et al.* (1988) with slight modification. Mixture containing extract solution (2 ml), phosphate buffer (2ml, 0.2M, pH 6.6) and 1% $K_3Fe(CN)_6$ (potassium ferricyanide 2ml, 10 mg/ml) were added and incubate for 20-30 min at 50°C. Tri chloro acetic acid (2ml, 100 mg/l) were added before centrifugation for short time. Finally the supernatant portion of each tube was transferred to 96 well plate followed by the addition of 0.1% ferric cyanide and distilled water. Spectrophotometric absorbance along with the standard was measured after 10 min at 700 nm. Increase absorbance of the reaction mixture indicates high reducing power.

Free radical scavenging assay (DPPH assay)

DPPH free radical scavenging activity was performed according to the method of Shimada *et al.* (1992) with slight modification. DPPH (2.5 mg) was dissolved in 100 ml methanol to prepare the stock solution and then stored at 20°C until needed. Dilution of DPPH solution with methanol is important to achieve specific absorbance of 0.980 at 517 nm with the help of spectrophotometer. DPPH solution of 180µl were dissolved with plant extract sample of 20µl in 96 well plate have 400µg/ml final concentration. Vitamin C was added as a positive control and absorbance of the reaction mixture and standard was measured at 717nm. Decrease in the absorbance of the mixture will show increase scavenging potential of the sample. Percentage radical scavenging potential of the sample was calculated by the given formula:

Percentage scavenging activity = $(A_0 - A_1)/ A_0 \times 100$

Where A_1 is the absorbance value of sample with DPPH solution while A_0 is the absorbance value of negative control.

Total phenolics content (TPC) estimation

The total phenolic contents of the given sample (4mg/ml) were estimated by the method described by Rehman *et al.*, 2014 with slight modification. Folin-Ciocalteu was employed as a reagent and gallic acid (4 mg/ml) was used as a positive control. A simple of 20µl with concentration of 4mg/ml was mixed with 90µl diluted stock solution of Folin-Ciocalteu reagent followed by 30 min of incubation. After the addition of 90µl of 6% sodium carbonate the absorbance of each sample along with the standard was measured at 630 nm with the help of spectrophotometer. Same procedure for sample and standard was repeated three times and the resultant total phenolics values were expressed as µg GAE (Gallic acid equivalent) per mg of dry plant extract.

Total flavonoids content (TFC) determination

Total flavonoids content estimation was measured by using an aluminum chloride based colorimetric method (Haq *et al.*, 2012). Formation of the aluminum chloride complex mixture containing 20µl of each sample (4mg/ml DMSO), 10µl of 1M potassium acetate buffer, 10µl of 10% aluminum chloride and 160µl of distilled water followed by incubation for 30 min at room temperature. Quercetin (4mg/ml) was used as a standard for plotting calibration curve. Absorbance of the reaction mixture and standard mixture was measured at 415nm wavelength and the final calculation of the total phenolics contents was expressed as µg equivalent per mg of the sample.

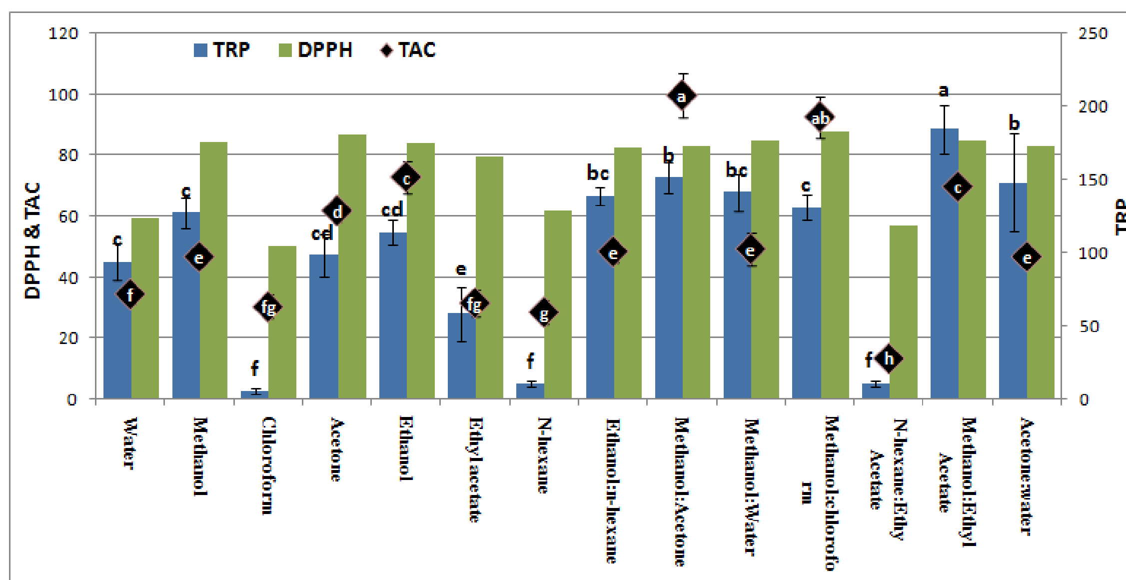

Fig. 1: Total antioxidant (TAC), DPPH radical scavenging (DPPH) and total reducing power (TRP) potential of *Fragaria* × *ananassa* leaves extracts. The results are presented as mean with standard deviation. The same letter shows no significant difference between mean values at significance level 0.05 using LSD

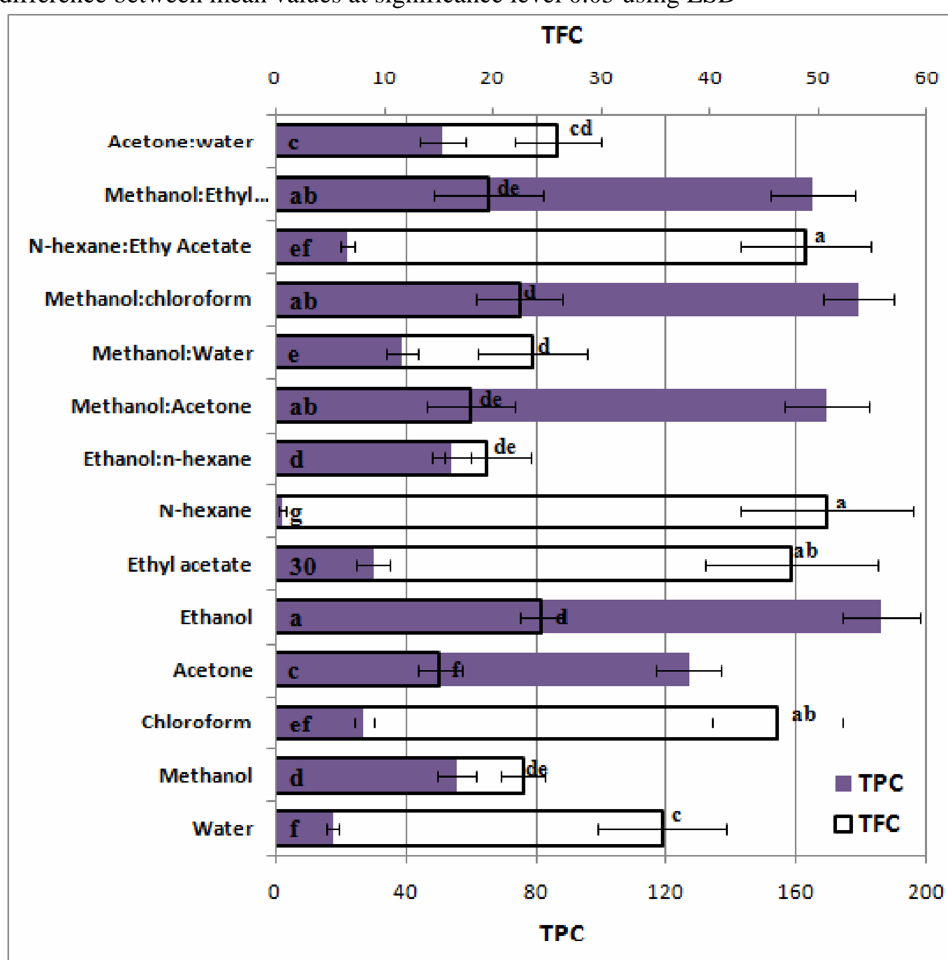

Fig. 2: Total phenolics (TPC) and total flavonoids (TFC) contents in *Fragaria* × *ananassa* leaves extracts. The results are presented as mean with standard deviation. The same letter shows no significant difference between mean values at significance level 0.05 using LSD.

Table 1: Cytotoxicity and protein kinase inhibition assay of *Fragaria × ananassa* leaves extracts

Extract	Bine shrimp assay (LD50 µg/ml)	Protein kinase assay (mm)
Aqu	51.00	8±0.7^{c}
MeOH	51.81	7±0.5^{d}
CHCl3	57.20	--
Acet	52.63	7±0.6^{d}
EtOH	53.70	8±0.7^{c}
Et.Ace	52.91	--
n-hex	52.05	--
EtOH+n-hex	50.99	8±0.7^{c}
MeOH+Acet	52.52	--
MeOH+Aqu	54.29	7±0.7^{d}
MeOH+CHCl3	53.45	15±1.2^{ab}
n-hex+Et.Ace	51.91	13±1.1^{b}
MeOH+Et.Ace	52.41	14±0.7^{b}
Acet+Aqu	52.03	16±0.7^{a}

The results are presented as mean with standard deviation. The same letter shows no significant difference between mean values at significance level 0.05 using LSD

Table 2: Antibacterial activity of *Fragaria × ananassa* leaves extracts against bacterial strains

Extracts used	*M. luteus*	*E. coli*	*S. aureus*	*B. bronchiseptica*	*Klebsiella*	*Pseudomonas*	*S .typhi*
Aqu	--	--	--	--	--	--	--
MeOH	9±0.9^{Ab}	11.5±1^{Ba}	6±0.6^{Dd}	7.5±0.5^{Ac}	--	9.5±0.7^{Bb}	9±0.8^{Ab}
CHCl3	9±0.8^{Abc}	19±0.8^{Aa}	11±1.2^{Ab}	7±0.6^{Ac}	7.5±0.7^{ABc}	11±1^{Ab}	6±0.4^{D}
Acet	8±0.7^{Ba}	--	--	--	--	7±0.6^{Db}	7±0.4^{Bb}
EtOH	9±0.8^{Ac}	19±0.8^{Aa}	11±1.2^{Ab}	7±0.6^{A}	7.5±0.7^{ABd}	11±1^{Ab}	6±0.4^{De}
Et. Ace	7±0.4^{Cb}	8±0.8^{Ca}	8±0.7^{Ba}	7±0.5^{Ab}	8±0.6^{Aa}	--	7±0.5^{Bb}
n-hex	7±0.4^{Cb}	8±0.6^{Ca}	--	--	--	8±0.5^{Ca}	7±0.5^{Bb}
EtOH: n-hex	--	--	--	--	--	--	--
MeOH: Acet	8±0.7^{Ba}	--	7±0.5^{Cb}	7±0.4^{Ab}	8±0.6^{Aa}	--	8±0.5^{Ba}
MeOH: Aqu	7±0.6^{Ca}	6±0.6^{Dc}	7±0.5^{Cb}	6.5±0.5^{Bc}	7±0.6^{Ba}	--	--
MeOH: CHCl3	--	--	--	--	--	--	--
n-hex: Et. Ace	8±0.5^{Ba}	6.5±0.6^{Dc}	8±0.5^{Ba}	--	7±0.7^{Bb}	7±0.4^{Db}	--
MeOH: Et. Ace	7±0.6^{Ca}	6±0.6^{Dc}	7±0.5^{Cb}	6.5±0.5^{Bbc}	7±0.6^{Ba}	--	--
Acet: Aqu	--	--	--	--	--	--	--

The results are presented as mean with standard deviation. The capital letters show LSD value with the column while the small letters show LSD value within the row. The same letter shows no significant difference between mean values at significance level 0.05 using LSD.

Brine shrimp cytotoxicity assay

The brine shrimp lethality assay was performed by the procedure as described Bibi *et al*. (2011a) with minor modification in 96 well polystyrene plate. Brine shrimp (*Artemia salina*) larvae were hatched at 37°C in a bi-partitioned tank filled with artificial sea water. Incubation temperature was kept 21 to 30°C. After two days the hatched mature nauplii were collected in a small beaker. Stock solution of 100mg/ml of each extract was further diluted into 250, 500 and1000 µg/ml concentration for lethality test. The mentioned concentration dilution was transferred into each separated well containing 20 nauplii and sea water addition with dried yeast. The death rate of these larvae against all dilution was observed and calculated. Negative control well adjusted, contain sea water and nauplii but no sample while positive control contain standard drug doxorubicin 4 mg/ml, sea water and nauplii. Final volume are adjusted and kept under fluorescence light at room temperature for 24 h. Test was repeated three times and the number of dead nauplii was counted in each well. LD_{50} was measured by comparing standard drug with percentage growth inhibition using table curve 2D ver.4 software.

Protein kinase inhibition assay

Streptomyces strain is used in protein kinase inhibition assay according to the procedure described by Fatima *et al*. (2015) with minor modification. Whole experiment

was carried out in sterile condition. Incubated streptomyces strain was inoculated into trypton soy broth and poured into Petri plates where the test organisms were grown. Filter paper discs were kept on the surface of prepared loan media and poured small amount of the sample and standard on that surface. Surfactin were used as positive control while DMSO was used as negative control. Incubation period was applied 24 to 36h for target strain growth development. Formation of bald or clear zone around the disc shows inhibition of spores and mycelia.

Antibacterial activity

Well diffusion methods for antibacterial assay were carried out according to the standard method of Bibi *et al.* (2011b) with slight modification. The bacterial strain (listed in table 2) was inoculated from the stored slant into nutrient broth (which has been adjusted to 0.5 McFarland standards). The prepared inocula were kept for incubation at 36°C for about 19-24h. Prepared nutrient agar was poured onto the sterile petri plates. When the media solidified, 0.1ml of the inocula containing 10^{6}cells/ml was poured onto the surface of prepared nutrient agar lawn and spread evenly with a sterile spreader. Well of 6 mm diameter was made with the help of sterile cork borer. The test sample of 10µl and 15µl in a concentration of 20 mg/ml was added into the well. Cefixime was used as positive control while DMSO was used as negative control. The Petri plates were incubate at 37°C for 24h. After the incubation, zone of inhibition was measured around each well.

STATISTICAL ANALYSIS

All the experiments were performed in triplicate. Results are reported as mean with standard deviation. Further the means were separated using least significant difference (LSD) at significance level 0.05.

RESULTS

Fragaria × ananassa leaves extract is evaluated for presence of phytochemicals and biological activities. The extracts prepared in different solvents based on polarities showed different level of activities. The biological activities where determine their nutritional values they are also helpful in medical applications and also isolation of respective component. Every solvent fractions of the strawberry leaves extracts showed excellent total antioxidant activity especially fraction of MeOH: Acet (207.65±6 µg AAE/mg) followed by MeOH: CHCl3 and EtOH (193.06±14, 152.08±7 µg AAE/mg, respectively) (fig. 1). The result shown in fig. 1 also report that maximum reducing power capacity was observed by MeOH: Et. Ace (88.58±20 µg AAE/mg) followed by MeOH: Acet, Acet: Aqu and MeOH: Aqu by giving the values 72.83±16, 71.22±16 and 68.01±16µg AAE/mg, respectively. DPPH free radical scavenging activity defines capturing of free radicals that might be lethal to cellular system. The maximum DPPH value was given by MeOH: $CHCl_3$ and Acet fractions (87.68%, 86.88% inhibition, respectively) of *Fragaria x ananassa* leaves extracts while the lowest values was given by $CHCl_3$ 50 % inhibition (fig. 1).

All the fractions also showed good result giving excellent values for total phenolic contents, ranges from 186±10 to 1.91±5µg AAE/mg (fig. 2). Maximum total Phenolic contents values was given by EtOH (186±10µg AAE/mg) followed by MeOH: Acet (169±13µg AAE/mg) and MeOH: Et. Ace (165±13µg AAE/mg) while the minimum values were given by n-hex and Aqu (1.91±5, 17.6±2µg AAE/mg, respectively). All the fractions also showed excellent total flavonoids contents as shown in the fig 2. Among all the fraction maximum value was given by n-hex (50.8±8µg AAE/mg) followed by n-hex: Et. Ace (48.8±6µg AAE/mg fraction), while lowest value was given by Acet (15.1±2µg AAE/mg).

In current study, most of the fractions have shown equality in their values by giving an excellent result, however significant cytotoxic activity was perceived by EtOH: n-hexan fraction (50µg/ml) of LD50 while lowest LD50 value was given by CHCl3 (57.20µg/ml) (table 1). All fractions of the leaves part were observed to have capability of inhibiting protein kinases but only four of the fourteen (Acet: Aqu, MeOH + CHCl3, MeOH: Et. Ace and n-hex: Et. Ace) have potent zone of inhibition as compared to others by giving values 16±0.7mm, 15±1.2mm, 14±0.7mm and 13±1.1mm respectively (table2) while the rest of the fractions have not potent zone of inhibition. Most of the fraction are active against each of the seven bacterial species (table 2) except fraction of Aqu, EtOH: n-hex, MeOH: CHCl3 and Acet: Aqu which have not shown any activity. Detailed results of each solvent are present in the table 2.

DISCUSSION

The total antioxidant capacity of the *F. x ananassa* extracts dissolving with different solvent can be ranked ascorbic acid equivalent. Every solvent fractions of the strawberry leaves extracts shows excellent antioxidant activity especially fraction of MeOH: Acet. Total antioxidant capacity (TAC) has been normally used to evaluate the antioxidant capacity of the extracts. During this kind of assay condition extracts can reduced Mo (VI) into Mo (V) and form a green colored complex known as phosphomolybdenum V complex which shows high absorbance at 695 nm (Jayaprakasha and Patil, 2007; Hossain and Rahman, 2011). The maximum absorbance value specifies that extract possess significant antioxidant activity. The reducing capability of different solvent extract of the plant was investigated ascorbic acid

equivalent by measuring the transformation of potassium ferricyanide (Fe^{3+}) to potassium ferro-cyanide (Fe^{2+}) following the method of Oyaizu *et al.* (1988). The results show that maximum reducing power capacity was shown by MeOH: Et. Ace followed by MeOH: Acet, Acet: Aqu and MeOH: Aqu. It is stated that the reducing power of the plant might be due to their hydrogen donating ability and accordingly, strawberry might contain higher amounts of reductone which could stabilize and block radical chain reaction by reaction with free radicals (Zhu *et al.,* 2015).

The DPPH of *F.* × *ananassa* extract was tested using a MeOH solution of the stable free radical, DPPH. Newly prepared DPPH solution with absorption maximum at 517 nm exhibit a deep purple color and when an antioxidant present in the solution, this purple color automatically fades or disappears. And thus more antioxidant capacity molecules can quench DPPH free radicals by providing hydrogen atoms or electron donation and as a result convert them into colorless product. This assay is generally employed for antioxidant studies of extract or specific compound across a short time scale. As described the maximum DPPH value was given by MeOH: $CHCl_3$ and Acet fractions (87.68%, 86.88% inhibition, respectively).

Total phenolics content estimation among various solvent fractions was represented in terms of Gallic acid equivalent using the standard curve equation. All the fractions shows good result giving excellent values, ranges from 186±10 to 1.91±5μg AAE/mg. Maximum by EtOH extract followed by MeOH: Acet. Total phenolics contents are one of the markers for the evaluation of oxidative stress (Inderjit and Nilsen, 2003). Phyto-constituents and other oxygenated phytochemicals possess significant antioxidant potential and free radical scavenging power. In the current result all the fractions shows different values as proposed by Fatima *et al.* (2015) that the extraction of phenolic compounds from the sample is directly related to the compatibility of the compound with the solvent system according to the 'like-dissolves-like' principle. Thus the present result expressed that plant phenolics exhibit a wide range of solubility in solvents with different polarity (Rice *et al*., 1997; Xie *et al.,* 2015). Total flavonoid content estimation were also evaluated as Gallic acid equivalent using standard curve equation with the same procedure as phenolic content valuation. All the fraction shows excellent total flavonoids contents. Among all the fraction maximum value was given by n-hex followed by n-hex: Et. Ace. Halli well, (2008) reported that plants rich in flavonoids inhibit lipid per oxidation because they are potential source of natural antioxidant that would add to the overall antioxidant capacity of an organism. Therefore, the current result suggests that flavonoids and phenolics may be the major contribution for the antioxidative stress and inhibitory action towards the oxidative reaction *in vitro* and *in vivo*.

Cytotoxic effect of each fraction was evaluated using brine shrimp lethality assay. Very feasible and simple method using a zoo-logic organism proved to be an appropriate method for screening in the discovery and monitoring of natural product. Larval tissues of shrimps respond similarly as mammalians carcinoma cells (Ullah *et al.,* 2012). Now a days, much attention is being given on anticancerous drug preparation by using natural sources, for this purpose plant extract are used to check their anticancer activity. In current study, most of the fractions have shown equality in their values by giving an excellent result, however significant cytotoxic activity was perceived by EtOH: n-hexan fraction. Hussain *et al*. (2007) reported that higher the cytotoxic potential of a plant can lead to provide cellular protection against necrosis and act as an immunological parameter to monitor disease progression. Protein kinase inhibitors are a well-established class of clinically useful drugs, particularly for the treatment of cancer. For particular protein kinases, achieving inhibitor selectivity often remains a significant challenge as a tool for chemical biology research or in the development of new small molecules as drugs (Smyth and Collins. 2009). In current study, all fractions of the leaves part were observed to have capability of inhibiting protein kinases. There has been no information about protein kinase inhibition assay of this plant and the result shows that this plant has the capability to inhibit protein kinases. Antibacterail activity was performed against seven different species to evaluate antibacterial potential of the strawberry plant. The plant activity was measured by calculating zone of inhibition around each disc in mm. Most of the fraction are active against each of the seven bacterial species except few fractions.

CONCLUSION

The study concludes that *F. x ananassa* leaves are also good source of potent antioxidative molecules along with antibacterial and protein kinase inhibition activities. The low cytotoxicity of plant extracts will diverge the attention to isolate bioactive constituents.

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