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Bioactive compounds, antioxidant and antifungal activity of medicinal plants of Western Ghats of Karnataka against Phytophthora blight disease of papaya (*Carica papaya* L.)

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Abstract
Aim of the present study was to analyse the bioactive compounds, antioxidant and associated antifungal activity against *Phytophthora* blight disease of papaya. The bioactive compounds and related antioxidant capacity of methanolic leaf extracts of four medicinal plants viz., *Santalum album, Nyctanthes arbor-tristis, Cinnamomum verum* and *Tridax procumben*s infusions were analysed. The antioxidant capacity was estimated by phosphomolybdic method, Ferric Reducing Antioxidant Power (FRAP) assay and DPPH assay. The primary metabolites like carbohydrate, protein, lipid and secondary metabolites like total phenolics, alkaloids, saponins, tannins and flavonoid contents were analysed quantitatively. Protein, phenolics, alkaloid, tannin and saponin contents were high in *Santalum album.* The flavonoids and carbohydrate contents were high in *N. arbor-tristis.* The antioxidant activity was high in *Nyctanthes arbor-tristis* and *Cinnamomum verum* and low in *Santalum album* and *Tridax procumben*s. The antifungal assay showed maximum zone of inhibition in higher concentration of all plant extracts (25µl-100 µl). The results of the present study showed that all the four plants have high potential antioxidant activity to fight against the *Phytophthora* blight disease of papaya.

Keywords: Antioxidant, antifungal, bioactive, *Carica papaya*, medicinal plants, Western Ghats

Introduction
Medicinal plants have been extensively studied for their antioxidant activity in recent years. It is believed that an increased intake of food rich in natural antioxidants is associated with lower risks of degenerative diseases, particularly cardiovascular diseases and cancer. Antioxidants from aromatic, spicy, medicinal, and other plants were studied to develop natural antioxidant formulations for food, cosmetic, and other applications. There are three major classes of plant chemicals: terpenoids, phenolic metabolites, and alkaloids. Among these three groups, phenolic compounds are the most important for dietary applications and the most extensively researched. Phenolic compounds include phenolic acids (hydroxybenzoic and hydroxycinnamic acids), polyphenols (hydrolyzable and condensed tannins), and flavonoids. Siddharthan, Yi-Zhong Cai *et al.,* (2007) [1]. These compounds protect plants, fruits, and vegetables from oxidative damage and have been used as antioxidants by humans. Finding new and safe antioxidants from natural sources is of great interest for applications in natural antioxidants, functional foods, agriculture and neutraceuticals. Phytochemical screening is one of the methods that have been used to explore antioxidant compounds in plants. Quy DiemDo *et al.,* 2014) [2].

Free radical biology is a discipline researched more broadly in life science. It mainly explores the formation and the scavenging of free radicals, as well as the damage caused by free radicals in biological systems. It is now well established that a series of oxygen-centred free radicals and other reactive oxygen species (ROS) contribute to the pathology of many disorders including atherogenesis, neurodegeneration, chronic inflammation, cancer and physiological senescence. Ani and Varadaraj *et al.* (2006) [3]. Therefore, antioxidants are considered important nutraceuticals on account of their many health benefits and they are widely used in the agriculture as potential inhibitors of lipid peroxidation. Scherer and Godoy (2009) [4].

Papaya is considered as one of the most important fruits because it is a rich source of various nutrients, including antioxidants. Like other plants diseases, papaya is also infected by many fungal, bacterial and viral pathogens which are the major problems to be managed in

agriculture. Various fungi cause rots in fruit of papaya. Among these diseases, *phytophthora* blight caused by *Phytophthora palmivora* is one of the most devastating diseases of papaya because it becomes established in wet, rainy weather when spraying is useless because the fungicides are washed away. Zhu and Agldayani *et al.,* (2007) [5], Sawant and Gawai (2011) [6].

Natural antioxidants may function as reducing agents, as free radical scavengers, as complexes of pro-oxidant metals and as quenchers of the formation of singlet oxygen. The most common natural antioxidants are flavonoids, cinnamic acid derivatives, coumarins, tocophenols and polyfunctional organic acids. The antioxidants of plants are phenolic. Yizhong Caiaand and Qiong Luob *et al.,* (2004) [7]. Hence, this attempt will be made by employing medicinal plant extracts to prevent the major fungal diseases in papaya (*Carica papaya* L.).

Phytophthora Simsa and Tjosvoldb *et al*., (2019) [8] is a cosmopolitan genus of Oomycete obligate plant pathogens containing approximately 60 described species. (Min Yang, and Shengchang Duan *et.al.,* (*2018*) [9] *Phytophthora* species attack a wide range of plants, and are responsible for some of the world's most destructive plant diseases - examples include the European potato famine of the 19th century caused by *P. Infestans.* Bevan and Weir *et. al.,* (2015) [10]. *Phytophthora* diseases have been well studied in the temperate regions of the world. However, *Phytophthora* diseases are very common throughout the wet tropical regions of the world and cause significant diseases losses in many tropical fruit crops in the form of root rots, collar rots, stem, leaf blights and fruit rot. For example, *P. palmivora* alone causes a myriad of severe diseases on many different crops including: black pod of cocoa; root, stem and fruit rot of pawpaw; root rot and blight of citrus; bud rot in palms; black stripe in rubber; and root rot, trunk canker, and fruit rot in durian.

Species of *Phytophthora* vary greatly in their degree of host specificity. *P. Fragariae* var. *rubi* infects a single host species. Gilles Berger and Katarzyna Czarnocka *et.al.,* (2015) [11] while *P. cinnamomi* is able to attack over 1000 different host plant species Gilles-Alex and Joseph Mpika *et.al.,* (2018) [12] and other species occupy a continuum between these two extremes. Broad host range *Phytophthora* species tend to attack their hosts using enzymes which affect relatively unspecialised host chemical and mechanical resistance mechanisms. Elizabeth and Bush *et al*., (2003) [13]. Whereas some host specific species are known to possess virulence genes which interact specifically, in a gene-for-gene system, with host resistance genes. (Li-Na and Wen *et al*., (2016) [14]. Hence, the present study focused on bioactive compounds, antioxidant and antifungal activity of medicinal plants against *Phytophthora* blight disease of papaya.

Materials and Methods

Collection of Plants and Sample preparation

Plants were collected from Western Ghats of Karnataka and brought to the laboratory in polythene bags. The plant samples were washed with running tap water and rinsed with distilled water and blotted died. Then the plant samples were oven dried at 40 ^{0}C and powdered and dried samples were extracted in methanol using Soxhlet apparatus and preserved for the further study. Vinitha Rani, Laveena *et al*., (2018) [15]. Ramya and Chandra (2018) [16].

Quantitative analysis of primary metabolites

Proteins

1 g of each dried and powdered samples were extracted by stirring with 50 ml of 50% methanol (1:5 w/v) at 25 °C for 24 h and centrifuged at 7,000 rpm for 10 min. 0.2 ml of extract was pipette out and the volume was made up to 1.0 ml with distilled water. To this 5.0 ml of alkaline copper reagent was added and allowed to stand for 10 min. 0.5 ml of Folin's-Ciocalteau reagent was added and incubated in dark for 30 min. The intensity of the colour developed was read at 660 nm. Lowry, Rosebrough (1951) [17]. Raffia Tazeen and Mular *et al.,* (2017) [18].

Total lipids

10 g of powdered sample was used to extract the lipids with 150 ml of petroleum ether for 16 hr at a solvent condensation rate of 2–3 drops/sec according to AACC Approved Method 30-25 with minor modifications of sample size and extraction time. The extract was concentrated and evaporated at room temperature to dry. The weight of extract was the total lipid content and it was expressed as mg/g dry matter. Cheung and Leung *et al.,* (2017) [19].

Carbohydrates

Determination of total soluble Carbohydrates the total soluble Carbohydrate content was determined according to the method described by Hedge and Hofreider (1962) [20]. 1 ml of sample was mixed with 4 ml of Anthrone reagent. Incubated in boiling water bath for 8 minutes, after which the absorbance was read at 630 nm against a reagent blank. The analysis was performed in triplicates and the results were expressed as mg / g sample. Geedhu Daniel and Sarun Mani (2016) [21].

Quantitative analysis of secondary metabolites

Total phenol

Total phenolic contents were determined by the Folin - Ciocalteau method. Ebrahimzaded *et al.,* (2008a & b) [22]; Nabavi *et al.,* (2008) [23] 0.5 ml of each of sample extracts was mixed with 5 ml of Folin- Ciocalteu reagent [1 ml FC reagent was diluted with 10 ml distilled water (1:10)] for 5 min and 4 ml 1M aqueous Na_2CO_3 (4 ml, 1 M) were then added. The mixture was allowed to stand for 15 min and the phenols were determined by colorimeter at 765 nm. The standard curve was prepared by 0, 50, 100, 150, 200, and 250 mg ml-1 solutions of gallic acid in methanol: water (50:50, v/v). Total phenol values were expressed in terms of Gallic acid equivalent (µg/g of dry mass) which is a common reference compound. Kamran Ghasemi *et.al.,* (2014) [24] .

Alkaloid

5 g of each powdered sample was weighed into a 250 ml beaker and 200 ml of 20% acetic acid in ethanol was added and covered to stand for 4 hrs. This was filtered and the extract was concentrated using a water bath to one-quarter of the original volume. Conc. ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected by filtration and weighed. Madhu and Sailaja *et al.,* (2016) [25].

Saponin
20 g of each plant sample was suspended in 200 ml of 20% ethanol. The suspension was heated over a hot water bath at 55 °C for 4 hrs with continuous stirring. The mixture was filtered and the residue re-extracted with another 200 ml of 20% ethanol. The combined extracts were reduced to 40 ml over water bath at about 90 °C. The concentrate was transferred into a 250 ml separator funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated and 60 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation, the samples were dried in the hot air oven to a constant weight. The saponin content was calculated in percentage. Obadoni and Ochuko, (2001) [26].

Total flavonoids
The total flavonoid content was determined by the standard method. Chang and Yang *et al.,* (2002) [27]. 0.5 ml of the sample was mixed with 1.5 ml methanol followed by 0.1 ml of 10% $AlCl_3$, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 30min, the absorbance of the reaction mixture was measured at 415 nm. The amount of flavonoid content was expressed as (µg/ml) equivalents of quercetin / mg of sample.

Tannin
20 µl of the sample was aliquoted into a test tube holding 980 µl of distilled water. 500 µl of 1% $K_3 Fe (CN)_6$ and 100 µl of 1% $FeCl_3$ were added and was made up to 3 ml with distilled water. After 10 minutes, the reaction mixture was measured ± using UV spectrophotometer at 720 nm. The tannin content was expressed as µg/ml of tannic acid equivalents/mg of extract. Sosuke and Yoshikazu, (2018) [28].

Total antioxidant assay
Antioxidant capacity was determination by Phosphomolybdate Method. The total antioxidant capacity of the extract fractions was determined by phosphomolybdate method using ascorbic acid as standard. To an aliquot of 0.05 ml (20-100 µg) of the extract solution prepared in DMSO, 0.25 ml methanol was added followed by the addition of 3 ml of reagent (0.6 M Sulphuric acid, 28 mM Sodium Phosphate and 4 mM Ammonium Molybdate). The tubes were capped and incubated in a water bath at 95 °C for 90 min, cooled to room temperature and the absorbance was measured at 695 nm. The total antioxidant capacity was expressed as µg of ascorbic acid equivalents per gram of sample (µg ascorbic acid /g of sample) by using the standard graph. The results were expressed as mean values. Mishra and Kumar *et al.,* (2011) [29].

Reducing power assay
Various concentrations (0.2-1.0 ml) of methanolic plant extracts were mixed with 2.5 ml of phosphate buffer and .5 ml of Potassium Ferricyanide. This mixture was kept at 50 ^{0}C in water bath for 20 mins. After cooling, 2.5 ml of 10% Trichloro Acetic Acid was added and centrifuged at 3000 rpm for 10 mins. From the supernatant, 2.5 ml solution was mixed in 2.5 ml distilled water and 0.5 ml freshly prepared ferric chloride solution. The absorbance was measured at 700 nm. Control was prepared in similar manner excluding samples. Ascorbic acid at various concentrations was used as standard. Increased absorbance of the reaction mixture indicates increase in reducing power. Shoib and Baba *et al.,* (2015) [30].

DPPH radical scavenging activity
DPPH free radical scavenging assay was measured using the method. Wong (2006) [31]. Various concentrations (0.1- 0.5ml) of methanol extracts were taken in a number of vials containing 3 ml of 0.1 mM methanolic solution of DPPH. The test tubes were shaken gently and set aside for 30 minutes at room temperature in dark. Optical density of samples was read at 520 nm against blank. Ascorbic acid was used as the standard control. All the tests were performed in triplicates. Free radical scavenging activity was expressed as inhibition percentage and was calculated using the following formula:

$$\text{Percentage inhibition (\%)} = (\text{O. D of Control} - \text{O. D of Sample}) / \text{O. D of Control X 100}$$

Culture and isolation of *Phytophthora* from papaya fruit
The diseased fruits were brought to the laboratory from the papaya plantation, Mangalore region. Fruits were sterilized in distilled water. Then infected area of fruit was cut into small pieces using sterile forceps and scalpel under Laminar Air Flow. Then the cut pieces of fruits surface were placed on the autoclaved Potato Dextrose Agar (PDA) media in the petriplates, and then incubated at 28 °C for 3- 5 days until the fungi proliferation on media to takes place. *Phytophthora* species was identified by referring the manual André Barbara (2001) [32]. Isolated pure fungal colony was streaked on fresh sterile PDA slants and incubated at 28 °C for 5-7 days and were maintained at 4 °C for further study. Rashad and Ahmed (2011) [33].

Antifungal assay
Agar well diffusion assay
The plant extract were allowed to diffuse out into the medium and interact in a plate freshly seeded with the *Phytopthora sp.* Petri plates containing 15 ml of PDA medium was solidified. To this, 0.1 ml of culture broth of *Phytopthora sp*. was inoculated using a sterile spreader (L-shape) on the plate surface to form a lawn of fungal growth to be observed. Wells were punchered using cork borer and 25, 50, 75 and 100 µl of the methanolic plant extracts were added. The plates were then incubated at 28 °C for 3-5 days. The diameter of zone of inhibition was measured in millimetres. Magaldi and Mata *et al.,* (2004) [34], Vinitha Rani and Chandra (2018) [35].

Determination of minimum inhibitory concentration (MIC)
The minimum inhibitory concentration was determined using methanolic extracts of plants which inhibit the visible growth of fungi. MIC is usually considered as the most basic laboratory measurement of the activity of antimicrobial agent against fungi. Different dilutions (25, 50, 75, and 100 µl) of the plant extracts were assayed against the test organisms. Distilled water was used as negative control. Then the tubes were incubated at 28 ^{0}C for 3-5 days. After incubation, MIC of each sample was determined by reading the optical density at 600 nm in UV spectrophotometer. The minimum inhibitory concentration was defined as the lowest concentration able to inhibit any visible fungal growth. (Jinglin and Sheena *et al.,* (2012); Scorzoni *et al.,* (2007) [36, 37]

Results and Discussion

Primary metabolites including Carbohydrates, proteins and lipids group of compounds, which are essential to life. Almost all organisms use carbohydrates as building blocks of cells and as a matter of fact, exploit their rich supply of potential energy to maintain life. Proteins are essential to maintaining the structure and function of all life and vital for growth and development. The presence of higher protein level in the plants towards their possible increase in food value or that a protein based bioactive compound could also be isolated in future. Laveena and Chandra (2017) [38].

Results of the primary metabolites of the medicinal plants depicted in the fig.1. The carbohydrate was found to be higher in *C. verum*, *N. arbor-tristis* and *T. procumbens* and lower in *S. album.* Carbohydrate content was high in *Catharanthus roseus*. Carbohydrates are one such group of carbon compounds, which are essential to life. Almost all organisms use carbohydrates as building blocks of cells and as a matter of fact, exploit their rich supply of potential energy to maintain life. Shirwaikar and Malini *et al.,* (2003) [39]. *S. album* showed high Lipid and protein content compared to *C. verum*, *N. arbor-tristis* and *T. procumbens.* The higher amount of plant lipid can be used as essential oils, spice, oleoresins and natural food colours. Plant lipids have developed products that work with diverse requirements, as culinary, medicinal and cosmetics. Proteins are found to be higher in *Abrus precatorius*, which are primary components of living organisms. Proteins are essential to maintaining the structure and function of all life and vital for growth and development. Bhumi and Savithramma (2014) [40].

Secondary metabolites analysis is necessary for extraction, purification, separation, crystallization, identification of various phytocompounds. Several studies have indicated that antioxidants prevent the onset of degenerative illness such as certain cancers, cardiovascular and neurodegenerative diseases, contracts, oxidative stress dysfunctions and aging. Renuga Devi and Krishnakumari (2015) [41].

Secondary metabolites of the selected medicinal plants were represented in fig.2. Total phenolic content was high in *S. album* compared to *C. verum*, *N. arbor-tristis* and *T. procumbens*. The presence of phenolic compounds in the plants indicates that these plants may be anti-microbial agent. Ofokansi *et al.,* (2005) [42].

Alkaloid content was maximum in *S. album*, *C. verum* and *N. arbor-tristis* and minimum in *T. procumbens*. Saponin content was found high in all the plants except *C. verum.* Saponin has the property of precipitating and coagulating red blood cells. Some of the characteristics of saponins include formation of foams in aqueous solutions, hemolytic activity, cholesterol binding properties and bitterness. Sodipo *et al.,* (2000) and Okwu, (2004) [43, 44]. Pure isolated alkaloids and their synthetic derivatives are used as basic medicinal agents for their analgesic, antispasmodic and bactericidal effects. Okwu (2004) [45]. They exhibit marked physiological activity when administered to animal. Flavonoids, on the other hand are potent water-soluble antioxidants and free radical scavengers, which prevent oxidative cell damage, have strong anticancer activity. The tannin content was high in all the plant extracts, whereas flavonoid was found to be higher in *N. arbor-tristis* compared to *S. album*, *C. verum* and *T. procumbens*. Herbs that contain tannins are astringent in nature and are used for treating intestinal disorders such as diarrhoea and dysentery. Tannins comprise both condensed non-hydrolysable tannins, known as proanthocyanidins, and esters of gallic acid and ellagic acid defined as hydrolysable tannins. Swahili and Knack (2004) [46], Puupponen, Nohynek *et al.,* (2005) [47].

Total antioxidant activity

Total antioxidant activity was maximum in *C. verum* and minimun in *N. arbor-tristis*, *S. album* and *T. procumbens* (fig.2). The phosphomolybdenum method is based on the reduction of molybdenum (VI) by the antioxidants and the formation of a green molybdenum (V) complex, which shows maximum absorbance at 695 nm. Preliminary Phytochemical Screening, Quantitative Estimation of Total Flavonoids, Total Phenols and Nidal and Fatima *et al.,* (2015) [48]. The plant extracts exhibited varying degrees of antioxidant capacity. Recent reports indicated that these medicinal plants extracts have strong antioxidant activity. Girennavar and Jayaprakasha, *et al.,* (2008) [49] could be attributed to the presence of flavonoids, polyphenols, and tannins. Mishra, *et al*., (2009) [50].

Reducing Power Assay

C. verum and *N. arbor-tristis* and *S. album* showed high reducing power activity and very low in *T. procumbens* with different concentrations (fig.3). Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity. Oktay *et al*., (2003) [51] Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid per oxidation processes, so that they can act as primary and secondary antioxidants. Chanda (2009) [52]. In this assay, the yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of each compound. Presence of reducers causes the conversion of the Fe^{3+} ferricyanide complex used in this method to the ferrous form. By measuring the formation of Pearl's Prussian blue at 700 nm, it is possible to determine the concentration of Fe^{3+} ion.

DPPH radicals scavenging

The DPPH radicals scavenging activity was high in *C. verum*, *N. arbor-tristis* and low in *S. album* and *T. procumbens* (fig.4). DPPH assay is one of the most commonly used methods for screening antioxidant activity of plant extracts. Joash and Yau (2015) [53]. It has been largely used as a quick, reliable and reproducible parameter to search the *in vitro* general antioxidant activity of pure compounds as well as plant extracts. Marja and Kahkonen *et al*., (1999) [54]. DPPH produces violet colour in methanol solution. It is reduced to a yellow coloured product, diphenylpicryl hydrazine. Antioxidants react with DPPH, a nitrogen-centered free radical, which is a stable, and converts it to α, α- diphenyl-*β*-picryl hydrazine. The degree of discoloration indicates the scavenging potential decrease in absorbance by the DPPH radical with increasing concentration of the extracts in dose dependent manner results in the rapid discolouration of the purple DPPH, suggesting that extracts of plants have radical scavenging antioxidant activity due to its proton donating ability. Adesegun and Fajana *et al.,* (2007) [55]. Reduction in the number of DPPH molecules can be correlated with the number of available hydroxyl groups. Several extract fractions exhibited significantly higher inhibition percentage (stronger hydrogen –donating ability) which can be positively correlated with total phenolic content. Singh and Chidambara *et al*., (2002) [56].

Antifungal assay

The antifungal assay was carried out by well diffusion method for preliminary assessment of inhibition activity. The extracts were tested against the *Phytophthora* blight disease of Papaya, in which the maximum zone of inhibition shown by all the extracts at highest concentration (25-100µl) (Table 1). Maximum inhibition zone was observed in all the four plants when tested against *Phytophthora* blight disease of papaya (fig.6). It also supports the earlier investigation Banso and Adeyemo (2007) [57] that the tannins isolated from the medicinal plants possess remarkable toxic activity against bacteria and fungi and may assume pharmacological importance. Varaprasad and Prasanth Kumar *et al.,* (2009) [58].

The Minimum inhibitory concentration showed that the less number of viable cells at higher concentration of *C. verum*, *S. album* and *N. arbor-tristis* where as *T. procumbens* extract showed moderate number of viable cells at all different test concentration (5 mg/L-50 mg/L)(fig.7). Minimum number of viable cells at higher concentration (5 mg/L-50 mg/L) was observed in *C. verum*, *S. album* and *N. arbor-tristis* followed by *T. procumbens.* Crude plant extracts are generally a mixture of active and non-active compounds. With more refined and solitary compounds, MICs of less than 100 mg/l may be suggestive of good anti-microbial activity. (Sader and Fedler *et al.,* (2004); Duncan and Pierre *et al.,* (2008) [59, 60].

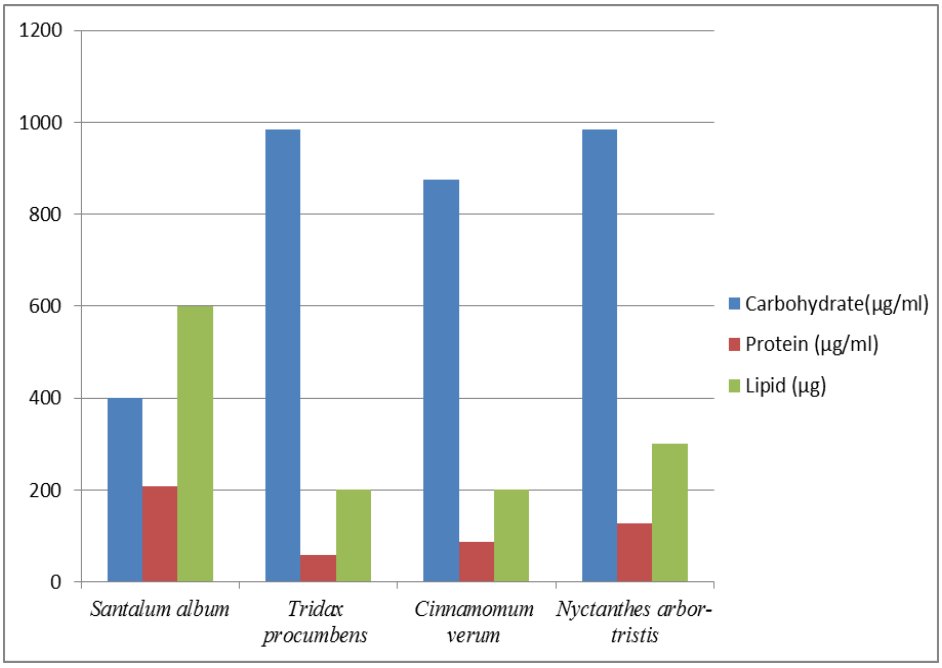

Fig 1: Quantitative analysis of primary metabolites

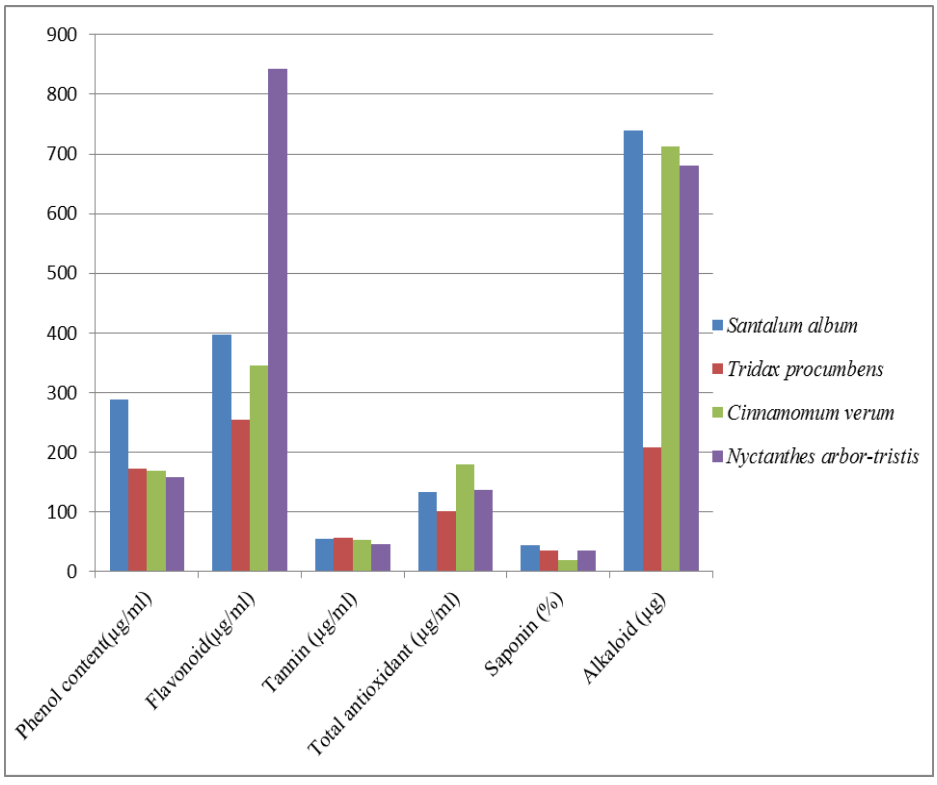

Fig 2: Quantitative analysis of secondary metabolites

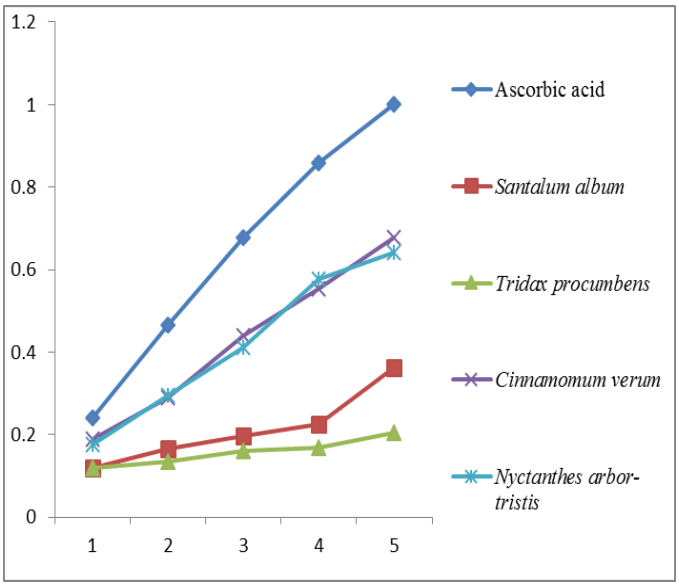

Fig 3: Determination of reducing power assay

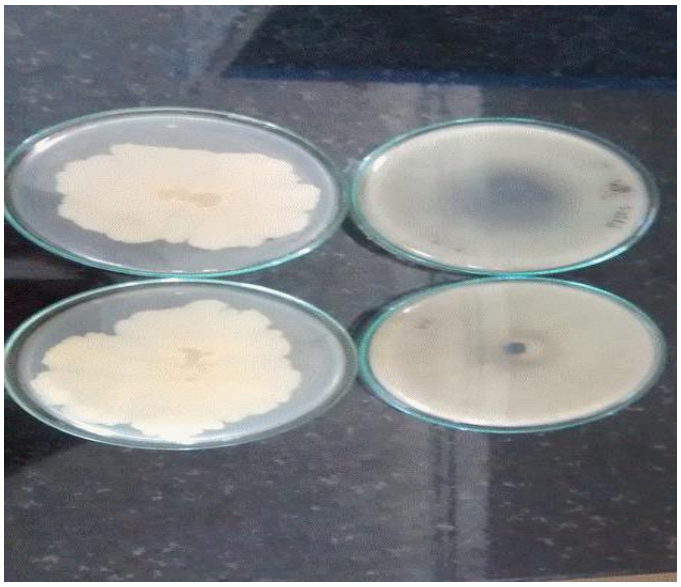

Fig 5: Isolated *Phytophthora sp.* of Papaya.

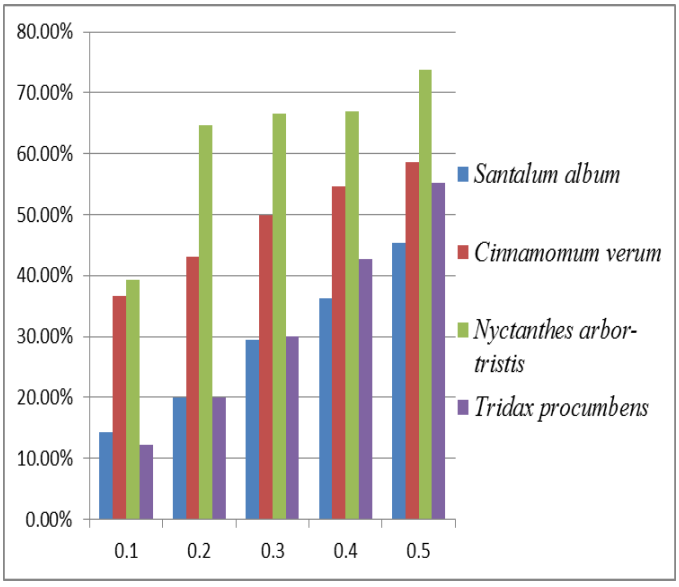

Fig 4: Determination of antioxidant by DPPH assay

Fig 6: Antifungal activity by well diffusion method

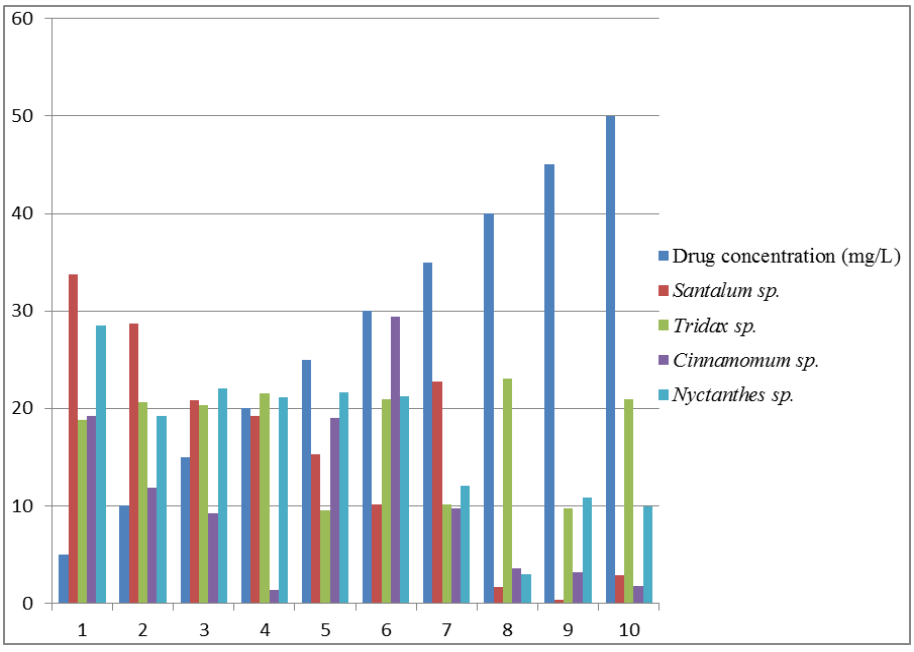

Fig 7: Determination of minimum inhibitory concentration (MIC)

Table 1: Antifungal assay by Agar well diffusion method

Conc. of Methanolic extract (µl)	Zone of inhibition (mm)			
	Plants			
	S. album	***N. arbortristis***	***C. verum***	***T. procumbens***
25	5.05± 0.208	11± 0.057	9.05± 0.1	6.1± 0.11
50	17.15±0.251	15.05± 0.1	10.1± 0.152	8.15± 0.152
75	19.15±0.212	20.1± 0.1	14.15±2.343	15.1± 0.152
100	19.25±0.5	25.15±0.152	22.05± 0.1	18.15±0.152

Conclusion

Western Ghats of Karnataka is very rich in medicinal wealth. The forest and hills of this region is a treasure of house of many medicinal plant species. The plants contain numerous molecules which are biologically active ingredients of potential drug to fight against diseases. Our results showed that the selected medicinal plants are rich in bioactive compounds and have high antioxidant capacity. All the plants showed antifungal activity against *Phytophthora* blight disease of papaya with higher concentrations.

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