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Influence of tree botanicals on seed germination and enzyme activity in blackgram (*Vigna mungo* L.) and cowpea (*Vigna unguiculata* L.)

K. Sangeetha, C. Swaminathan*, E. Subramanian, B. Sivasankari[1] and P. Kannan[2]
Department of Agronomy, LBNF laboratory, Agricultural College and Research Institute,
Tamil Nadu Agricultural University, Madurai-625104, Tamil Nadu, India
E.mail: brownrevolution@yahoo.com

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ABSTRACT

In petri plate bioassay we studied the effects of aqueous leaf extracts from 3- tree species *viz*., mango (*Mangifera indica* L.), sugar apple (*Annona squamosa* L.), moringa (*Moringa oleifera* L.) on seed germination and seedlings growth of blackgram (*Vigna mungo* L.) and cowpea (*Vigna unguiculata* L.). We also identified the bioactive compounds in these extracts. The aqueous 5 % leaf extract of *A. squamosa* significantly increased the germination and seedling growth of both blackgram and cowpea. The seed germination was stimulated by 15 % and 0 %, root length by 21 % and 36.6 %; shoot length by 30.9 % and 21.77 %, seedling weight by 25 % and 95 %than control, respectively. Aqueous leaf extract of *M. oleifera* at 5 % concentration also improved the shoot length; root length and seedling dry weight in both crops over control. On the contrary, it inhibited the seed germination of cowpea by 15 %, when compared to control. Besides, allelochemicals found in moringa leaves also inhibited the germination. According to GCMS results, 2-piperidinone (26.50 %), catechol (15.50 %) and benzofuran, 2,3-dihydro (21.48 %) were, respectively, abundant in the leaf extracts of Annona, Mango, and Moringa. The presence of high amount of phenolic and flavonoids compounds in moringa leaves inhibited the cowpea seed germination than annona. Thus, tree botanicals could be used as a biostimulant as well as bioherbicide to replace the synthetic agrochemicals, thereby, protecting the environment.

Key words: *Annona squamosa*, bioassay, blackgram, cowpea, GC-MS, germination, *Mangifera indica*, mango, Moringa, *Moringa oleifera*, seedling growth, sugar apple, *Vigna mungo, Vigna unguiculata*.

INTRODUCTION

Both blackgram (*Vigna mungo* L.) and cowpea (*Vigna unguiculata* L.), family fabaceae, are important pulse crops with high nutritional value (40). The use of present pesticides (Weedicides, insecticides, nematicides, fungicides) have caused several problems in Agriculture, hence, there is need for innovative non-chemical products to boost food production and quality (10). Spread of organic farming across the globe offers great scope for alternatives to synthetic agrochemicals to promote crop growth as natural biostimulants (plant-derived substances). These encourage seed germination, seedlings growth, resistances to biotic (25,37,48) and abiotic stresses (7,14,58), with little or no adverse effects.

Seed soaking before sowing mitigates detrimental effects of high temperatures and ensures enough moisture for faster seed germination. Different agrochemicals like growth promoting agents, fertilizers, pesticides, fungicides are used for seed treatment to protect the seeds from pests and to induce germination and better establishment. However, in recent times various natural products and botanicals have been used for this purpose. *Annona squamosa* has been employed as a natural medicine and in a wide range of distinct food

*Correspondence author, [1]Department of Mathematics, [2]Department of Soil Science.

uses, such as its pulp could be used as an ice cream flavouring agent and the fruit contains 50-80% edible portion, which can be pulped to make juice. It has significant vitamin C content of 35 to 42 mg per 100 g, as well as notable amounts of dietary fibre, vitamin B1 (thiamine), and potassium (56). The *Annona squamosa* leaf extract at 5 % concentration is used as foliar spray in irrigated cotton (11) due to its wide range of active phytochemicals.

Mango (*Mangifera indica*) leaves contains vitamins A, B, E, and C as well as minerals like nitrogen, potassium, phosphorus, iron, sodium, calcium, and magnesium (30). The mango phytochemicals (secondary metabolites) regulates the water-nutrients interactions, heat responses in plants and improve seed germination, root growth, chlorophyll accumulation, photosynthesis, and photo-assimilates translocation (44).

Moringa (*M. oleifera*) leaves and other plant organs aqueous extracts have the plant anti-dyslipidemic, anti-oxidant, anti-diabetic and chemo protective properties (8). Moringa leaf extract 5 % concentration is a cheap biostimulant than costly synthetic growth boosters (1,24,28). The presence of minerals, antioxidants, hormones and cytokinin, auxins and gibberellins in its leaf extracts makes it suitable for seed treatment (6). Besides it protects the cells from ageing by improving the antioxidant activities of several enzymes (57).

Contrarily, aqueous extracts from coir pith (*Cocos nucifera* L.) inhibits seed germination and growth of blackgram and cowpea (36) and Eucalyptus aqueous extracts inhibits *Chenopodium album* and *Portulaca oleracea* (3). The inhibition is due to the phytotoxins present in their extracts (42). Thus, identification of phytochemicals present in leaf extracts is necessary using GCMS (20) to know the scientific reasons for their negative or positive effects. This study aimed (i) to test the influence of botanicals as biostimulants on seed germination and seedlings growth of black gram and cowpea and (ii) to identify the principal compounds causing positive or inhibitory effects of these donor tree leaf extracts.

MATERIALS AND METHODS

The lab experiments were done in Seed Science Laboratory, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Madurai, Tamil Nadu, India (90.93 ^{o}N, 78.12 ^{o}E and altitude: 147 msl). The leaves of test trees were collected during March, 2022. The collected leaves were washed in tap water to remove dirt and ground with distilled water in 1:2 ratio. The extracts were filtered through fine mesh and pressed carefully for complete extraction that served as a stock solution. From this stock solution, 5 % concentration of leaf extract (v/v) was prepared with distilled water (51) and its pH and EC were directly determined using pH meter and EC meter. The allelopathic effects of donor tree leaf extracts *viz*., *Moringa oleifera*, *Mangifera indica* and *Annona squamosa* were tested on seed germination and seedlings growth of blackgram cv. VBN 8 and cowpea cv.VBN 3.

pH and EC of aqueous extracts

The solubility of nutrients and their interactions with other components in water are affected by pH and EC. A variation in plant sensitivity to pH and EC is observed even during seed germination. In this study, 90-100 % seed germination was observed in a pH range of 5.0-7.0 and an EC range of 0.3-0.4 dsm-1 in spray solution. The pH of foliar sprays should be slightly acidic (~5.8) and the EC less than 1 dSm-1 for the solution to penetrate the cuticle quickly and be absorbed by the leaves (due to complicated electrostatic repulsion and attraction phenomena within the cuticle by pH).

I. Lab. Bioassay

(i). Germination: It was done in petri dishes (9 cm dia) lined with germination papers (Fig 1). Ten unsterilized seeds each of blackgram and cowpea were placed separately on the germination paper. To keep the germination papers moist, on alternative days 2 mL leaf extracts of respective tree species were added per petri plate. Distilled water served as control. Petri-plates were kept in germination chamber (25 ± 2 °C temp and 95 ± 3 % RH) in dark. Test crops germination was recorded daily.

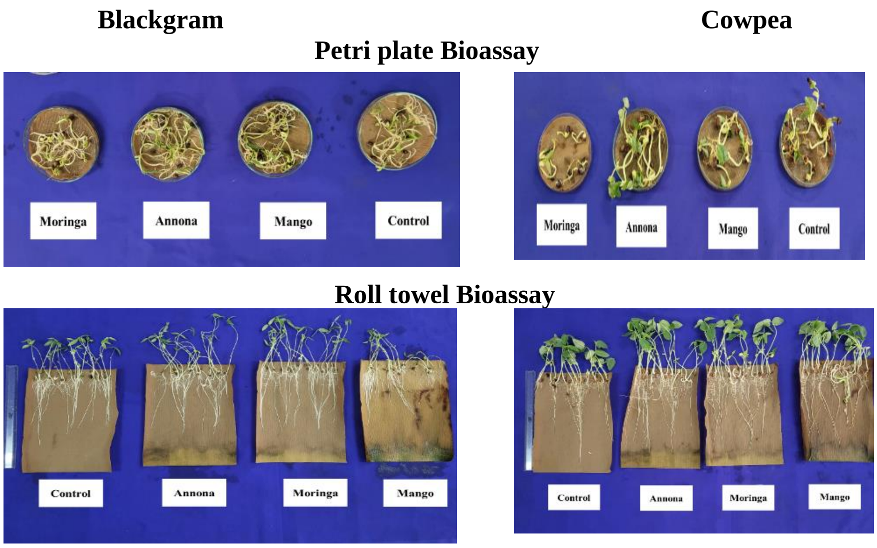


Figure 1. Petri plate bioassay and Roll towel bioassay on blackgram and cowpea

(ii). Seedlings growth: In a separate experiment, as per ISTA **(**23), roll towel method was used to assess the root length, shoot length and seedling weight. For this, 25 seeds each of blackgram and cowpea were placed on germination paper, wetted with respective tree leaf extracts and then the germination papers were rolled on. The rolled germination papers were immersed vertically in pre-sterilized beakers and half-filled (250 mL) with respective tree leaf aqueous extracts. and these extracts were replaced daily (40). Distilled water served as control. Both experiments were done in CRD (Completely Randomized Design) with 3-replications in germination chamber (25 ± 2 °C temp and 95 ± 3 % RH). In roll towel method experiment, seedling growth (root length, shoot length and seedling dry weight) of 5-random seedlings were recorded. The intensity of inhibition or stimulation in the bioassay germination and seedling growth were compared (45).

Germination indices

Different indices of germination [Germination (%), shoot length, root length, vigour index I (germination % x total seedling length) and vigour index II (germination % x total dry weight of seedling)] based on the daily germination data were calculated (2). Other germination parameters (i) Germination Energy (GE), (ii) Germination Value (GV), (iii)

Emergence Energy Value (EEV) and (iv) Germination Relative Index (GRI) were calculated as under:

Using a single index, does not accurately describe the allelopathic effects on the germination process. A comparison of different indices can make allelopathy a more accurate science.

(i). Germination Energy : The Germination Energy (GE) measures the seed ability for faster germination (54) and was calculated as under (33) :

$$GE = \frac{X1}{Y1} + \frac{X2-X1}{Y2} + \ldots\ldots\ldots\ldots + \frac{Xn-Xn-1}{Yn}$$

Where, X_n : Number of germinant on nth counting date; Y_n : Number of days from sowing to nt^{h} count.

(ii). Germination value: It is directly related to survival of seedlings and was calculated as under (13,15) :

$$GV = (\sum DGS / N) \times \frac{GP}{10}$$

Where, GV : Germination value, GP : Germination (%) end of test, DGS : Daily germination speed obtained by dividing cumulative germination percent by number of days since sowing, ∑ DGS : Summation of all DGS figures, N : Number of daily counts effective from date of first germination.

(iii). Emergence Energy Value (EEV, 9): The seed germination capacity and germination energy decides the early emergence, establishment and survival of seedlings. It represents both the emergence of the seed and the energy within the seed. If the seed has a higher germination energy this will be reflected in its early seedling emergence with higher values. Germination (%) is calculated daily, this is then divided by the number of days since the start of test. This calculation is done daily until the end of test. The highest obtained value is EEV.

(iv). Germination Relative Index (GRI, 50): It reflects the germination (%) on each day during the germination phase. Higher GRI values indicate higher and faster germination. It was calculated as under:

$$GRI = [\sum Xn\ (h - n)]$$

Where, Xn : Number of germinant at nth count, h : Total number of counts and n : Count number.

(v). Response index (RI): RI is used to compare the magnitude of inhibition v/s stimulation in bioassays (45). A negative RI reflects the proportional disparity in output (germination, radicle length or plumule growth), a positive RI reflects the stimulation in output of test crop over control. It was calculated as under:

if $T > C$, $RI=1-(C/T)$
if $T = C$, then $RI = 0$
if $T < C$, then $RI= (T/C) -1$

Where, T : Treatment mean and C : Control mean. A negative RI reflects proportional disparity in output (germination, radicle length or plumule growth) of test crop in treatment relative to output in control.

II. Biochemical analysis

(i). Dehydrogenase (Based on OD value)

A sample of 1.0 g seeds was soaked in water for 12 h in three replicates. Seed glumes were removed and incubated in dark with 5.0 mL of 0.2 % Tetrazolium chloride for 3 h. After incubation, the excess solution was decanted and the seeds were thoroughly washed with distilled water and surface dried with blotters. Formazan was eluted by soaking the stained seeds in 5 mL Methyl Cellosolve (2 methoxy ethanol) for 1 h and the optical density was measured using UV spectrophotometer at 470 nm (29).

(ii). α- amylase activity (units/g)

Two replicates of 500 mg of pre-germinated seeds were homogenized in 1.8 mL of cold 0.02 M sodium phosphate buffer (pH 6.0) and centrifuged at 20,000 rpm for 20 min to extract enzymes. For 0.1 mL of enzyme extract, one mL 0.067 % starch solution was added. After 10 min of incubation at 25 ^{o}C the reaction was stopped by adding 1.0 mL of iodine hydrochloric acid solution (60 mg potassium iodide and 6 mg iodine in 100 ml of 0.05N hydrochloric acid). Change in colour was measured at 620 nm by UV-VIS spectrophotometer (Model SP-2205). The activity was calculated and expressed as mg maltose min^{-1} (43).

α- amylase activity = (OD value / Volume of sample pipetted out) X (1000 / 500)

(iii). Catalase activity (units/g)

The seeds catalase activity was calculated by following modified method (32). To assess catalase activity, seeds were pre-conditioned by soaking in distilled water for 3 h. One g of embryos was ground in a pestle and mortar with phosphate buffer (20 ml of 0.067 M) by dissolving KH_2PO_4 (3.522 g) and $Na_2HPO_4\ 2H_2O$ (7.268 g) in distilled water and the volume was made up to 1.0 L (Assay buffer diluted 10 times) at 4 ^{0}C and centrifuged at 15000 rpm for 5 min. The supernatants were used for enzyme assay. In experimental cuvette, 3 ml of H_2O_2 phosphate buffer (0.16 ml of H_2O_2 (10 % w/v) diluted to 100 ml with phosphate buffer-prepared fresh) and 0.02 mL of sample (1.0 mL of sample diluted to 10 mL) were added and mixed well with a glass rod. The time (Δt) required for decrease in absorbance was noted at 240 nm in UV spectrophotometer. Catalase activity was calculated as under:

$$\text{Catalase activity} = \frac{17 \times 10 \times 20 \times 1000}{\Delta t \times X \times Y} \times 100$$

Where, Δt: Time required to decrease the absorbance, X: Volume of enzyme extract, Y: Volume of buffer solution.

(Iv). Peroxidase Activity (μg G^{-1})

The peroxidase activity of seeds was determined by following modified method (34). Seeds were pre-conditioned as described elsewhere. One g embryos were homogenized in pestle and mortar with 3.0 mL of 0.1 M phosphate buffer (pH 7). The homogenate was centrifuged at 10000 rpm for 10 min. The supernatant was the enzyme source. For 3.0 mL of H_2O_2 (0.142 mL of H_2O_2 diluted to 100 mL), 0.1 mL of enzyme extract (sample) was added. The time (Δt) required for increase in absorbance was noted with UV spectrophotometer at 436 nm. All procedures were done at 5 ^{0}C. Peroxidase activity was expressed as units/g of sample and calculated as under :

$$\text{Peroxidase activity } (\mu g\ g^{-1}) = \frac{3.18 \times 0.1 \times 1000}{6.39 \times W \times \Delta t \times X \times 1000}$$

Where, Δt : Time required for increasing the absorbance, X : Volume of enzyme extract, W : Weight of sample.

III. Milli-Q water extraction and GC-MS analysis

5.0 g powdered leaf sample was extracted with 50 mL milli-Q water in a mechanical shaker at 250 rpm for 3 days continuously. To concentrate the sample, the extracted material was filtered through Whatman No. 40 filter paper and kept in boiling water bath. To re-dissolve this sample, 2 mL of methanol was used and then filtered through a Paul membrane filter by injecting the sample into 2 ml syringe. The sample was used for GC-MS analysis.

IV. Gas-chromatography -Mass spectrometry (GC-MS) analysis

GC-MS analysis was done using GC-MS QP 2020 coupled with MSD. The GC fortified with Rxi-5 Sil MS fused silica capillary column (30mL x 0.25 D x 0.25 µm thickness). Helium was used as a carrier gas at a constant flow rate of 1 mL/min and keeping the ion source temperature and the injection temperature at 250 ºC. The device was initially adjusted to a temperature of 70 °C and kept for 5 min. The oven temperature was increased to 300 ºC at the conclusion. The mass spectra of compounds in samples were obtained using a detector that operated in scan mode between 40 and 650 m/z. The MS took 5 min to start and 51 min to finish, with a solvent cut time of 5 min.

Statistical Analysis

All data were statistically analyzed and the treatment means were compared using LSD (Least Significant Difference) at the probability level of both 1 % and 5 %.

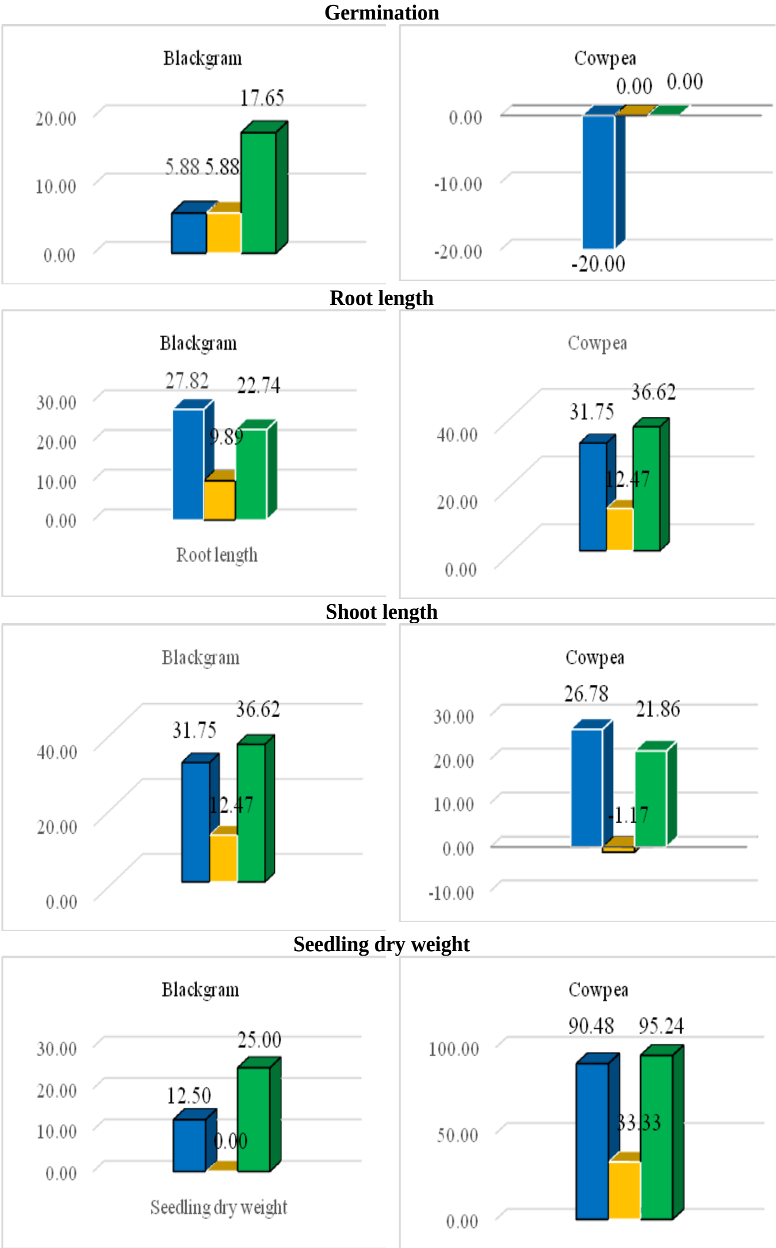
RESULTS AND DISCUSSION

Germination and seedlings growth

All donor trees leaf extracts stimulated the germination of blackgram, however, the Annona leaf extracts showed stronger stimulation (15 % increase) over the control (Fig. 2). In contrast, these extracts were inhibitory to the germination of cowpea seeds. Moringa leaf extract reduced the cowpea seed germination by 20 %. A higher germination (%) determines the seed volume and viability (39), it was favoured by the presence of active ingredients (19), micronutrients (35); biochemical compounds [phenolic compounds, organic acids, proteins, and alkaloids (47)] in leaf extracts. This supports the current findings. Both Annona and Moringa leaf extracts strongly stimulated the growth attributes (root length, shoot length, seedling dry weight, and vigour indices) in both crops. However, mango leaf extract showed both stimulatory and inhibitory effects in attributes of both test crops. The germination inhibition may be due to the presence of inhibitory phytochemical compounds in moringa leaves (36,40).

Response Index

Germination and related attributes of both crops were stimulated by tree leaf extracts, except the cowpea seed germination (Fig 3). The leaf extracts from 3-donor species stimulated the germination of blackgram as measured by RI means of 5.6 to 15 %. Contrarily these extracts inhibited the cowpea germination up to 20 % in Moringa leaf extract. Nevertheless, the three leaf extracts strongly stimulated the root and shoot growth in both crops. The stimulation in root growth was from 9 to 21.7 % in blackgram and 11.1 to 26.8 % in cowpea. The corresponding figures for shoot growth were 1.7 to 23.6 %



Moringa oleifera leaf extract @ 5 %, *Mangifera indica* leaf extract @ 5 %, *Annona squamosa* leaf extract @ 5 %

Figure 2. Effects of tree leaves aqueous extracts on germination and seedling growth of Blackgram and Cowpea

(blackgram) and -1.1 to 21.1 % (cowpea). Therefore leaf extracts stimulated the germination and seedlings growth in blackgram and were less or non-inhibitory in cowpea. Although leaf extracts inhibited the cowpea seed germination, but stimulated the seedlings growth (root and shoot length and seedling dry weight). Maximum seedlings growth stimulation (48.8 %) was observed in cowpea and 46.8 % in blackgram. Regarding vigour indices, the stimulation as measured by RI means ranged from 3.5 to 35 % in SVI and 3.5 o 48.8 % in SVII. Both inhibition and stimulation may be due to phytocompounds present in donor trees leaf extracts. The phytocompounds present in the tree extracts, were inhibitory to blackgram but were more inhibitory to cowpea.

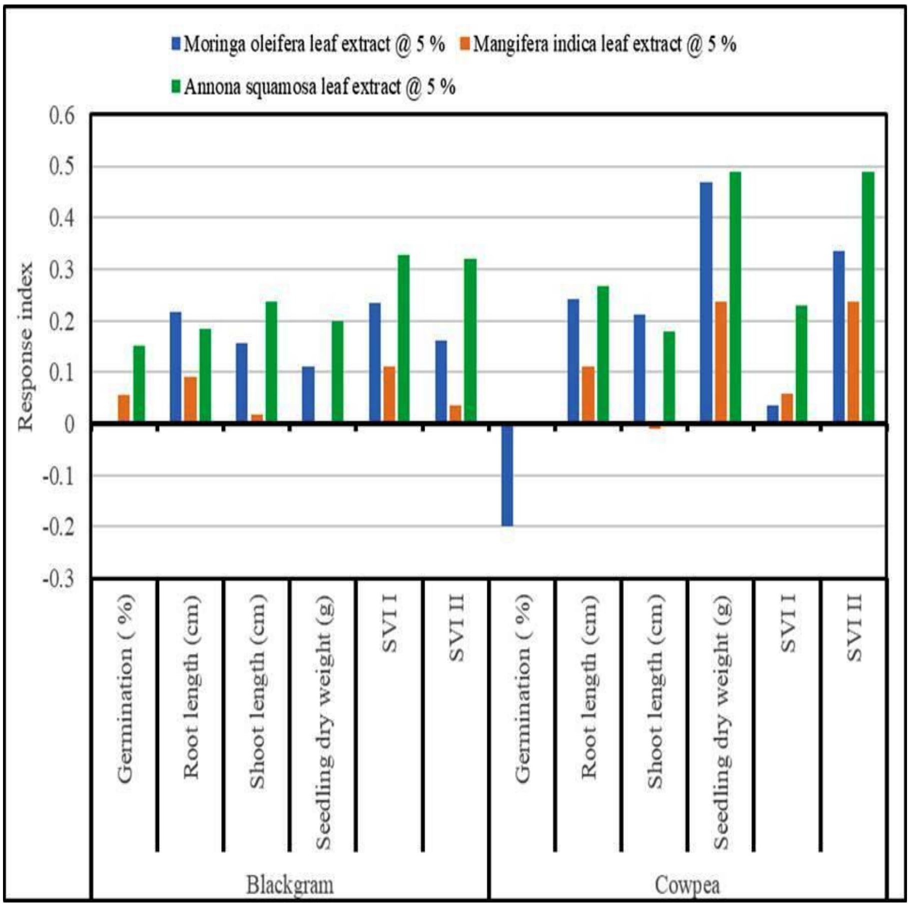


Figure 3. Response index for germination and seedling growth of Blackgram and Cowpea

Germination attributes

Germination energy influences the fast germination of seeds and the impact on the viability and vigour of seedlings (23). Among tree leaf extracts (Fig. 4). Annona extract had positive influence and maximized germination energy of 3.09, 3.33, as well as stipulated

Germination Values of (13.25, 14.29), Emergence Energy value (2.00, 3.33) and Germination Relative Index (66.5, 70.0). This could be due to the presence of diverse phytochemicals present in tree leaf extracts, including phytosterols, glycosides, essential oils, saponins, phenols, and flavonoids (49) which act as a precursor of GA_3 due to saponins and presence of terpenoids, phytosteroids, fatty acids and glycosides (47).

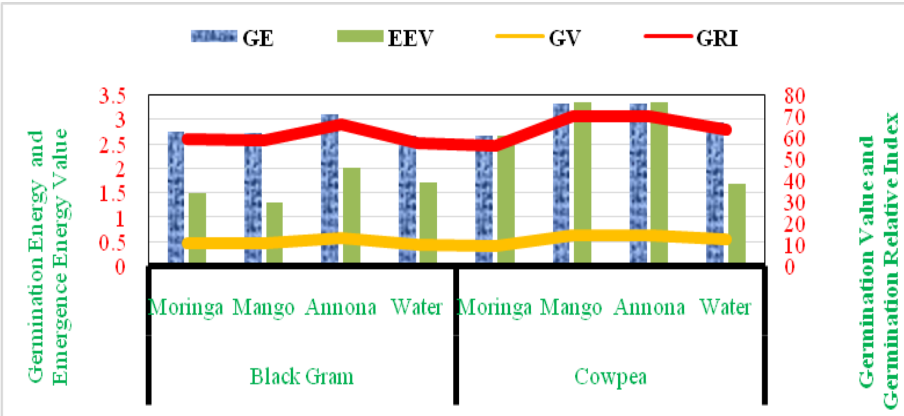


Figure 4. Effects of leaf extracts of 3-donor trees on germination attributes in Blackgram and Cowpea

The inhibition/stimulation effects cannot be studied with a single germination index as it would not show the allelopathic effects on germination. Therefore, different germination related indices were determined and the results showed the stimulation/inhibition effects of leaf extracts on both crops. The effects of tree leaf extracts on seed germination of both crops were stimulatory, except the mango leaf extract.

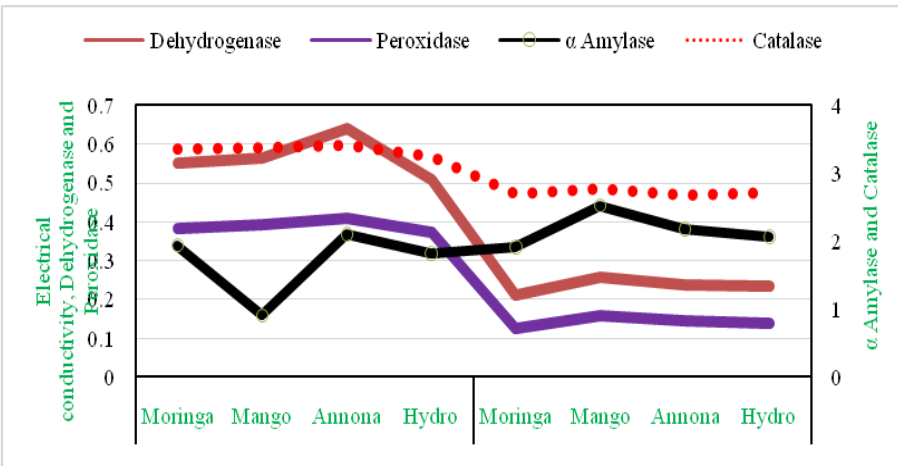


Figure 5. Influence of 3-donor trees leaf extracts on enzyme activities

Biochemical changes in seeds

This study revealed higher enzyme activity of dehydrogenase, catalase, peroxidase and α amylase were found in seed fortification with botanicals/leaf extracts (Fig. 5). Fortification decreases sugars and enhances the amylase activity that facilitates mobilization of reserves from seed storage to embryo, resulting in greater early seed vigour. This has been found in application of moringa leaf extract to maize (4,7) as higher dehydrogenase and peroxidase activity resulted in increased seedling vigour. This might be due to the presence of growth-promoting compounds that boosted respiration rate, showing involvement of antioxidant enzymes in crop growth (41).

GC-MS studies

The principal compounds found in tree leaves were identified by GC-MS analysis for scientific explanation of our findings by revealing the chemistry behind the beneficial influence of tree leaf extracts on seed germination and associated attributes (Tables 1,2,3).

Table 1. Identified bioactive compounds with Milli-Q water extraction of *M. oleifera* by GC-MS analysis

S. No	RT	Area (%)	Compound	Formula	MW	Chemical class
1.	5.021	0.37	Oxirane, [(1-methylethoxy) methyl]	$C_6H_{12}O_2$	116	Epoxides
2.	6.045	2.19	1H-Azepine, 1-acetylhexahydro-	$C_8H_{15}NO$	141	Azepanes
3.	8.547	0.65	1,2,3-Butanetriol	$C_4H_{10}O_3$	106	Secondary alcohols
4.	9.145	1.98	Valeric acid, 3-tridecyl ester	$C_{18}H_{36}O_2$	284	Fatty alcohol esters
5.	9.464	11.92	2-Pyrrolidinone	C_4H_7NO	85	Pyrrolidones
6.	9.562	4.78	2,5-Dimethylfuran-3,4(2H,5H)-dione	$C_6H_8O_3$	128	Furanones
7.	9.628	3.15	2-Pyrrolidinone	C_4H_7NO	85	Pyrrolidones
8.	9.703	0.57	p-Cresol	C_7H_8O	108	Phenols
9.	9.970	7.56	Benzeneethanamine	$C_8H_{11}N$	121	Phenethylamines
10.	12.571	26.50	2-Piperidinone	C_5H_9NO	99	Piperidinones
11.	13.464	9.33	Catechol	$C_6H_6O_2$	110	Phenols
12.	13.581	8.74	Catechol	$C_6H_6O_2$	110	Phenols
13.	14.601	0.70	1,2-Benzenediol, 3-methoxy-	$C_7H_8O_3$	140	Phenols
14.	15.798	1.03	Phenol, 4-(methoxymethyl)-	$C_8H_{10}O_2$	138	Benzylethers
15.	18.005	2.37	2-Furanmethanol, 5-ethenyltetrahydro-.alpha, 5-trimethyl-, cis-	$C_{10}H_{18}O_2$	170	Tetrahydrofurans
16.	18.098	3.21	trans-Linalool oxide (furanoid)	$C_{10}H_{18}O_2$	170	Tetrahydrofurans
17.	18.391	2.11	4-Ethylcatechol	$C_8H_{10}O_2$	138	Phenols
18.	18.933	0.56	Pyrrolidine, 1-[2-(1,3-cyclopentadien-1-yl)ethyl]-	$C_{11}H_{17}N$	163	Pyrrolidines
19.	20.833	2.68	Benzeneacetonitrile, 4-hydroxy- $$ Acetonitrile, (p-hydroxyphenyl)-	C_8H_7NO	133	Benzyl cyanides
20.	22.933	0.66	3-Methyl-4-phenyl-1H-pyrrole	$C_{11}H_{11}N$	157	Pyrroles
21.	25.102	1.88	7-Oxabicyclo[4.1.0]heptan-3-ol, 6-(3-hydroxy-1-butenyl)-1,5,5-trimethyl-	$C_{13}H_{22}O_3$	226	Oxepanes
22.	27.383	0.90	2-pyrrolidinone, 1-(4-hydroxyphenyl)-	$C_{10}H_{11}NO_2$	177	Phenylpyrrolidines
23.	29.354	2.37	1-(7-Hydroxy-1,6,6-trimethyl-10-oxatricyclo [5.2.1.0(2,4)]dec-9-yl)ethanone	$C_{14}H_{22}O_3$	238	Oxepanes
24.	31.483	1.27	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	Long chain fatty acids
25.	32.297	2.54	Oxazole, 4-ethyl-4,5-dihydro-2-(2-hydroxyphenyl)-	$C_{11}H_{13}NO_2$	191	Phenols

RT: Retention Time, MW: Molecular Weight

The chromatogram area indicated quantification of each component based on the area (%). Total 25 compounds were identified from each species using GC-MS spectra with Milli-Q water extraction. Moringa leaf had 25 components, the abundant phytochemicals were, 2-Piperidinone, 2-Pyrrolidinone, Catechol, Catechol and Benzene ethanamine with 26.50 %, 11.92 %, 9.33 %, 8.74 % and 7.56 % with retention time

Table 2. Identified bioactive compounds with Milli-Q water extraction of *M. indica*by GC-MS analysis

S. No	RT	Area (%)	Compound name	Formula	Mol Wt	Chemical class
1	5.021	0.57	2-Isopropoxyethylamine	$C_5H_{13}NO$	103	Dialkyl ethers
2	9.035	8.78	1,2-Cyclohexanediol	$C_6H_{12}O_2$	116	Cyclohexanols
3	13.241	15.50	Catechol	$C_6H_6O_2$	110	Phenols
4	13.375	11.13	Catechol	$C_6H_6O_2$	110	Phenols
5	13.475	3.03	[1,1'-Bicyclopropyl]-2-octanoic acid, 2'-hexyl-, methyl ester	$C_{21}H_{38}O_2$	322	Fatty acid esters
6	13.585	0.53	Benzofuran, 2,3-dihydro-	C_8H_8O	120	Coumarans
7	13.841	3.39	cis-4-Hydroxycyclohexanecar boxylic acid lactone	$C_7H_{10}O_2$	126	Lactones
8	14.820	2.72	6-Nitroundec-5-ene	$C_{11}H_{21}NO_2$	199	Organic nitro compounds
9	14.890	4.76	Cyclohexanone, 2-pentyl-	$C_{11}H_{20}O$	168	Carbonyl compounds
10	15.672	1.16	1,1'-Bicyclopentyl-1,1'-diol	$C_{10}H_{18}O_2$	170	Cyclopentanols
11	15.805	2.01	D-Verbenone	$C_{10}H_{14}O$	150	Monoterpenoids
12	15.969	1.14	[1,1'-Bicyclopentyl]-2-one	$C_{10}H_{16}O$	152	Carbonyl compounds
13	16.875	0.91	1-Butyl(dimethyl)silyloxy-1-cyclopentylethane	$C_{13}H_{28}OSi$	228	Organosilicon compounds
14	16.986	15.05	Cyclohexanone, 2-pentyl-	$C_{11}H_{20}O$	168	Carbonyl compounds
15	17.527	11.84	2-Dodecylcyclobutanone	$C_{16}H_{30}O$	238	Carbonyl compounds
16	17.861	1.16	trans-13-Octadecenoic acid	$C_{18}H_{34}O_2$	282	Long-chain fatty acids
17	19.499	1.14	Acetic acid, trifluoro-, octahydro-4-hydroxy-1,5-methano-1H-inden-1-yl ester (1.alpha.,3a.beta.,4.beta.,5.beta.,7a.beta.)	$C_{12}H_{15}F_3O_3$	264	Organoheterocyclic compounds
18	19.665	3.18	Cyclohexanol, 3,3,5-trimethyl-, acetate, cis-	$C_{11}H_{20}O_2$	184	Carboxylic acid derivatives
19	19.730	1.31	2-Cyclohexen-1-one, 3,5,5-trimethyl-4-(3-oxobutyl)-	$C_{13}H_{20}O_2$	208	Sesquiterpenoids
20	20.304	2.12	4-(1,3,3-Trimethyl-bicyclo[4.1.0] hept-2-yl)-but-3-en-2-one	$C_{14}H_{22}O$	206	Carbonyl compounds
21	22.112	2.68	1-Tetradecanamine, N,N-dimethyl-	$C_{16}H_{35}N$	241	Organonitrogen compounds
22	22.809	0.71	Tapentadol	$C_{14}H_{23}NO$	221	Phenylpropanes
23	23.313	3.76	1-Heptanamine, N,N-dimethyl-	$C_9H_{21}N$	143	Organonitrogen compounds
24	30.547	0.80	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	Fatty acid methyl esters
25	34.611	0.60	Phytol	$C_{20}H_{40}O$	296	Diterpenoids

RT: Retention Time, MW: Molecular Weight

Table 3. Identified bioactive compounds with Milli-Q water extraction of *A. squamosa*by GC-MS analysis

S. No	RT	Area (%)	Compound name	Formula	Mol Wt	Chemical class
1	5.021	0.30	5-Hexen-2-ol, 5-methyl	$C_7H_{14}O$	114	Alcohols and polyols
2	13.375	6.02	Catechol	$C_6H_6O_2$	110	Phenols
3	13.550	21.48	Benzofuran, 2,3-dihydro	C_8H_8O	120	Coumarains
4	13.840	3.840	cis-4-Hydroxy cyclo hexane carboxylic acid lactone	$C_7H_{10}O_2$	126	Cyclohexanols
5	14.085	1.16	Methylphosphonic acid, 2TMS derivative	$C_7H_{21}O_3PSi_2$	240	Organic phosphonic acids and derivative
6	15.808	1.59	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	150	Phenols
7	18.354	3.93	4-Ethylcatechol	$C_8H_{10}O_2$	138	Phenols
8	18.490	1.98	Pyrrolidine, 1-(1-cyclohexen-1-yl)	$C_{10}H_{17}N$	151	Pyrrolidines
9	19.408	13.13	Benzeneethanol, 4-hydroxy	$C_8H_{10}O_2$	138	Phenols
10	21.808	2.07	1-Butanone, 1-(4-aminophenyl)	$C_{10}H_{13}NO$	163	Alkyl-phenylketones
11	22.697	10.18	9-Oxa-bicyclo[3.3.1]nonane-1,4-diol	$C_8H_{14}O_3$	158	Oxanes
12.	23.351	1.25	6-Acetyl-4,4,7-trimethylbicyclo[4.1.0] heptan-2-one	$C_{12}H_{18}O_2$	194	Carbonyl compounds
13.	24.410	7.63	Quinic acid	$C_7H_{12}O_6$	192	Alcohols and polyols
14.	24.711	2.17	2,4a,8,8-Tetramethyl decahydro cyclo propa[d] naphthalene	$C_{15}H_{26}$	206	Sesquiterpenoids
15.	24.820	1.37	2-Butanone, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)	$C_{13}H_{22}O$	194	Sesquiterpenoids
16.	25.453	0.97	Spirio-10-(2,11-dioxabicyclo [4.4.1] undeca-3,5-diene)-2'-(oxirane), 1,3,7,7-tetramethyl-	$C_{14}H_{20}O_3$	236	Dioxepines
17.	25.887	2.10	2-Cyclohexen-1-one, 4-(3-hydroxybutyl)-3,5,5-trimethyl	$C_{13}H_{22}O_2$	210	Sesquiterpenoids
18.	27.383	2.84	2-pyrrolidinone, 1-(4-hydroxyphenyl)	$C_{10}H_{11}NO_2$	177	Phenylpyrrolidines
19.	27.566	1.45	2,6,10,10-Tetramethyl-1-oxaspiro[4.5] decan-6-ol	$C_{13}H_{24}O_2$	212	Tetrahydrofurans
20.	27.994	6.84	(S,E)-4-Hydroxy-3,5,5-trimethyl-4-(3-oxobut-1-en-1-yl)cyclohex-2-enone	$C_{13}H_{18}O_3$	222	Sesquiterpenoids
21.	29.390	2.63	2,3a-Dimethylhexahydrobenzofuran-7a-ol	$C_{10}H_{18}O_2$	170	Isobenzofurans
22.	30.544	0.63	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	Fatty acid methyl esters
23.	34.306	1.31	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	296	Fatty acid methyl esters
24.	43.639	1.83	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	$C_{19}H_{38}O_4$	330	Fatty acid esters
25.	44.689	1.30	(Z)-3-(pentadec-8-en-1-yl)phenol	$C_{21}H_{34}O$	302	Phenols

RT: Retention Time, MW: Molecular Weight.

of 12.571, 9.464, 13.464, 13.581 and 9.970, respectively. The peak bioactive compounds Mango, Maringa, Sugar maples were as under (Fig 6,7,8). In *Mangifera indica* leaf based on area percentage were Catechol (15.50 %), Cyclohexanone, 2-pentyl- (15.05 %), 2-Dodecylcyclobutanone (11.84 %), Catechol (11.13 %) and 1,2-Cyclohexanediol (8.78 %) with retention time of 13.241, 16.986, 17.527, 13.375 and 9.035 minutes. The peak bioactive compounds found in *A. squamosa* leaf extracts based on area (%) were Benzofuran, 2, 3-dihydro (21.48 %) with a retention time of 13.550 min, Benzeneethanol, 4-hydroxy (13.13 %) with a retention time of 19.408 min, and 9-Oxa-bicyclo[3.3.1]nonane-1,4-diol (10.18 %) with a retention time of 22.697 min.

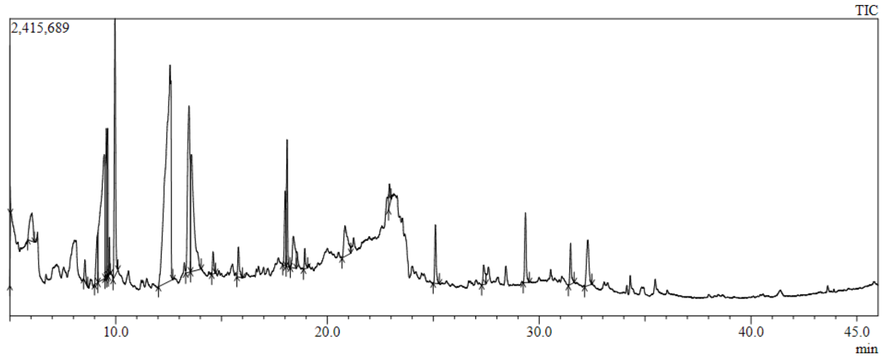


Figure 6. GC-MS Chromatogram of *M. oleifera* leaf

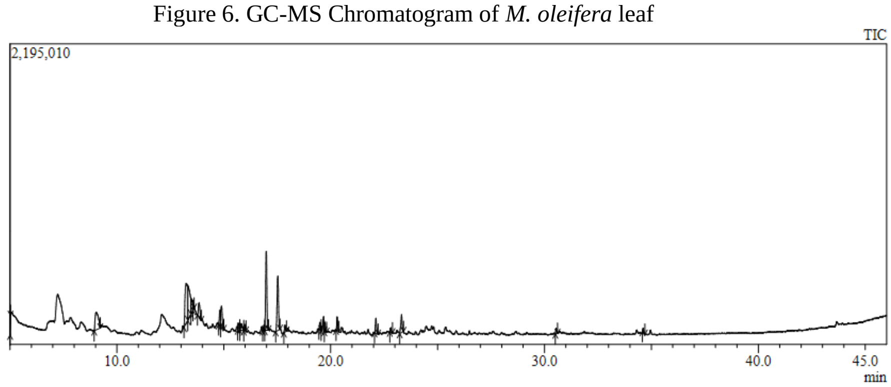


Figure 7. GC-MS Chromatogram of *M. indica* leaf.

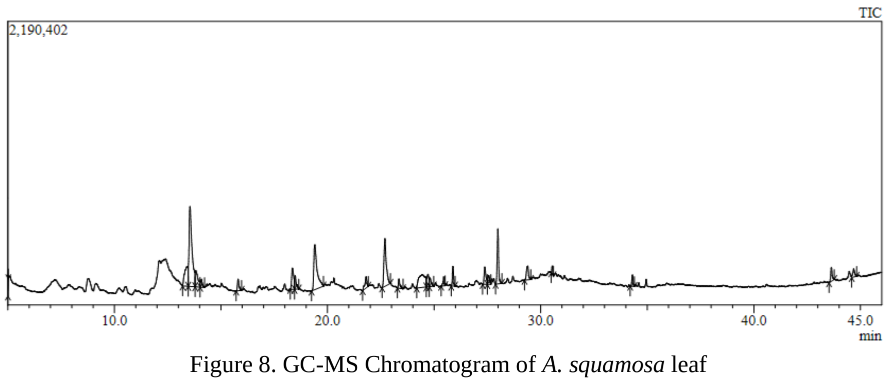


Figure 8. GC-MS Chromatogram of *A. squamosa* leaf

Among the identified compounds, alkaloids (32 %) and phenolic compounds (24 %) were the major secondary metabolites in moringa leaf (Fig 9). Similarly, mango leaf contained 24 % phenols and 32 % other derivative compounds and annona leaf contained 25 % phenolics, 16 % terpenoids and 12 % fatty acids (Fig. 10). These physiologically active compounds may be developed for plant-based foliar nutrition and plant protectants. The aqueous extract of *Annona* significantly increased the germination attributes of both crops

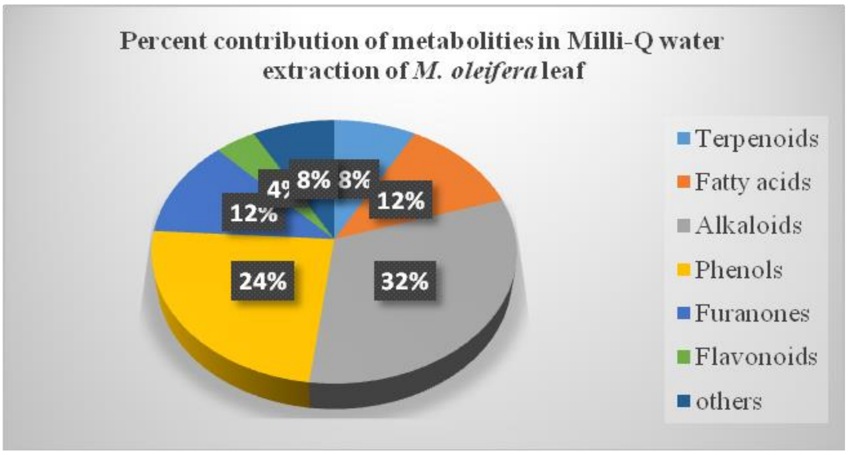


Figure 9. Contribution of extracted secodary metabolities from *M. oleifrea* on seedlings growth

than two other leaf extracts. This could be due to the presence of high amount of benzofuran, which has a wide range of biological properties (analgesic, anti-inflammatory, antibacterial, antifungal, anticancer and antiviral) (Fig 11). In last 10-years, benzofuran compounds have contributed 3.8 % to agriculture (38). Mango is rich in catechol phenol, it has stimulatory or

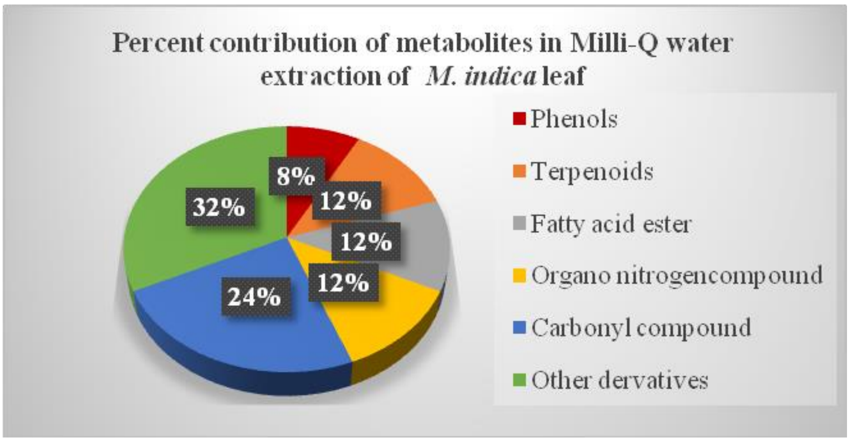


Figure 10. Contribution of extracted secodary metabolities from *M. indica* on seedlings growth

inhibitory effects on other crops (Fig 10). This is also consistent with the findings (53) that catechol is involved in auxin biosynthesis and regulates redox homeostasis in plants. In contrast, it is a strong weed inhibitor, as evidenced by the reduced seedling fresh weight especially in field poppy, but has only a minor effect on wheat and barley growth (52). The study findings (26) claimed that flavonoids in mango leaves have herbicidal properties against parthenium weed.

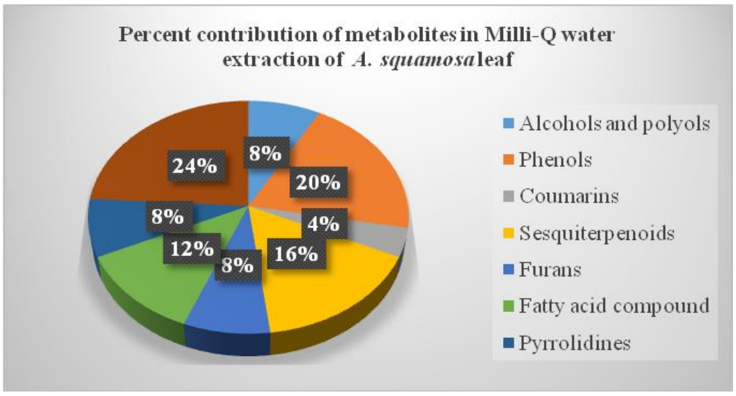


Figure 11. Contribution of extracted secodary metabolities from *A. squamosa* on seedlings growth

Both quantitative and qualitative phenolic compounds impacts the seed germination (31). Moringa leaves are rich in phenolic and flavonoid compounds, which might be the cause decreased germination of both cowpea and blackgram. These results were supported by the finding of (18) that showed that the amount of polyphenols in moringa leaf extract was more than two folds that of seed and root extract. Additionally, a significant number of flavonoids are present in the leaf extract of *M. oleifera*. These results support the possibility of allelopathic inhibition may be caused by phenols, flavonoids, or both (3,17).

M. oleifera used at lower doses increased the yield (12), whereas, at higher concentrations it inhibited germination and growth (21). Likewise, the leaf extract of *M. oleifera* can be used as bioherbicide, because the results are encouraging for both weed control and promoting the growth of main plants (16). Terpenoid compounds present in leaves, contributes significantly in plant defence against biotic and abiotic stresses. The terpenoids are the most abundant compounds found in *Glochidion ellipticum* leaves, followed by alkaloid compounds and esters of fatty acid compounds, and they play an important role in the life of most organisms (27). This study proves that the oldest Indian agricultural system relied on plants phytochemicals in tree leaves for food production, and thus positive allelopathy should be developed and studied further to produce high-quality food grains without the use of present pesticides, herbicides and agrochemicals.

CONCLUSIONS

The *Annona squamosa* leaf extract at 5 % concentration stimulated the seed germination and seedling growth of blackgram (*Vigna mungo* L.) and cowpea (*Vigna unguiculata* L.). But aqueous leaf extract of *Moringa oleifera* were inhibitory to germination in both crops. Seed germination stimulation showed positive allelopathic activity. The GC-MS study detected the abundance of 2-piperidinone (26.50 %), catechol (15.50 %), and benzofuran, 2,3-dihydro (21.48 %), respectively in the leaf extracts of Annona, Mango and Moringa. After more research, such tree botanicals could be developed as bio-stimulants to improve the seed germination and seedling growth to replace synthetic agents.

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DECLARATION

We declare that all authors of this manuscript made a significant contribution, and we have not excluded any author that substantially contributed. We have followed the ethical norms established by our respective institutions.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL APPROVAL

The authors declare that the study was carried out following scientific ethics and conduct. However, this study did not involve any use of animals, hence no ethical approval has been obtained from the concerned committee.

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